

ESTROGEN REGULATION OF MESSENGER RNA
STABILITY

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ABSTRACT

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ABSTRACT

Title of Dissertation: Estrogen Regulation of Messenger RNA Stability

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Estrogen administration to male Xenopus laevis results in the inhibition of serum albumin gene expression through the destabilization of albumin mRNA. This process occurs during the massive induction and stabilization of vitellogenin mRNA in the liver, and is mediated through an estrogen receptor-dependent process. This dissertation examines: 1) potential estrogen-induced changes in albumin mRNA primary sequence leading to its destabilization; 2) the possibility that other serum protein-coding mRNAs are coordinately regulated by estrogen; 3) the effect of metabolic inhibitors on the decay process; 4) the intracellular localization of the degradation; and 5) RNA degradation through the development of an in vitro assay system.

Estrogen does not modify the primary sequence of albumin mRNA in regulating its stability. This conclusion was based on comparison of albumin cDNA sequences determined from libraries prepared from RNA isolated from control versus estrogen-treated animals. Since the albumin cDNA sequences were identical, unique secondary structures caused by estrogen-treatment could not be responsible for targeting albumin degradation.

To investigate whether other serum proteins were similarly regulated by estrogen, several serum protein cDNAs were tested for coordinate post-transcriptional regulation of their cognate mRNAs, and their primary and secondary structures were analyzed. From this study it was determined that estrogen also destabilized mRNAs encoding γ -fibrinogen, transferrin, clone 12B, and the second protein of inter- α -trypsin inhibitor. Messenger RNA levels for these serum protein-coding mRNAs decrease to low levels in the cytoplasm in response to estrogen with little effects on steady-state RNA levels in the nucleus. Ferritin mRNA (encoding an intracellular protein) levels were unaffected upon estrogen-treatment. Comparisons of primary sequence and secondary structure of the 5' and 3' untranslated regions of estrogen-regulated mRNAs failed to reveal any gross sequence or structural similarities that could possibly target their degradation.

A cultured liver cube system was employed to determine whether protein synthesis was required for the degradation of albumin mRNA. Destabilization of albumin mRNA occurred in the absence of protein synthesis. Transcriptional activity increased after exposure to estrogen and was temporally correlated with the decrease in albumin and increase in vitellogenin mRNA levels.

To further localize the site of cytoplasmic albumin mRNA degradation, post-mitochondrial extracts were fractionated on sucrose gradients. The results suggested that albumin mRNA was being degraded on 20-80S mRNP fractions prior to its reaching the polyribosomes. The mRNP fractions contained little albumin mRNA after estrogen treatment. In contrast, the distribution of ferritin mRNA was unchanged while vitellogenin mRNA was mainly distributed on heavier polyribosomes only in extracts from estrogen-treated animals. The data are consistent with the notion that mRNP complexes are

important in differentiating albumin and other serum protein-coding mRNAs from ferritin mRNA in the degradation process.

An in vitro assay system was developed to study estrogen-regulated mRNA stability. Total RNA, synthetic [^{32}P]-labeled transcripts and mRNP particles were used as substrates. Nuclease activity was found in polyribosome fractions separated on sucrose gradients and degraded albumin mRNA faster than ferritin mRNA. The nuclease activity could be extracted with salt and further purified by phosphocellulose chromatography. The nuclease activity was hormone regulated; polysomes from estrogen-treated animals contained greater activity than corresponding fractions from untreated animals. This is the first such in vitro assay system to study hormonally-regulated RNA stability. Further development of this system should allow the biochemical purification of factors controlling RNA stability.

... to the Faculty of the Department of Pharmacology
... program of the University of the
... fulfillment of the
... the degree of
... 1977

ESTROGEN REGULATION OF MESSENGER RNA STABILITY

by

John E. Moskaitis

Dissertation submitted to the Faculty of the Department of Pharmacology
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requirements for the degree of
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DEDICATION

To my parents, Joseph and especially my mother Stella, and to other parents like them whose ambitions, dreams, and potentials may never be realized, but whose lives have been dedicated to seeing that their children's are.

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***** Please note in the Figure legends where other members of the Schoenberg laboratory participated in certain experiments. Their help is greatly appreciated. *****

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Abbreviations and Definitions

AmpR- ampicillin resistance gene

AMV reverse transcriptase- Avian Myeloblastosis Virus reverse transcriptase, RNA-dependent DNA polymerase that uses single-stranded RNA or DNA as a template to synthesize the complementary strand.

apo VLDL- apoprotein very low density lipoprotein

bp- base pairs

BSA- bovine serum albumin

CAT- chloramphenicol acetyltransferase

cDNA- complementary deoxyribonucleic acid

Church buffer- 0.5M sodium phosphate, pH 7.0; 1 mM EDTA; 1% BSA; 7% SDS

Coon's medium- modified Ham's F12 (0.65X)

CsCl- cesium chloride

DTT- dithiothreitol, a reducing agent used to prevent the formation of disulfide bonds.

E. coli- Escherichia coli

Eco RI linkers- 5'-CCGAATTCGG- 3' suitable for use in cloning
3'-GGCTTAAGCC- 5' procedures requiring insertion of
restriction sites into DNA.

EDTA- ethylenediamine-tetracetic acid, metal chelator

HEPES- (4-[2-hydroxymethyl]-1-piperazine-ethanesulfonic acid)

hnRNP- heterogeneous ribonucleoprotein particle

hsp- heat shock protein

Klenow- large fragment of DNA Polymerase I. DNA-dependent DNA polymerase that lacks the 5'→3' exodeoxyribonuclease activity of intact DNA polymerase I, but does contain the 3'→5' exodeoxyribonuclease activity.

LacZ- β -galactosidase gene

MDMP- 2-(4-methyl-2,6-dinitroanilino)-N-methylpropionamide; initiation inhibitor of protein synthesis.

Molecular Markers-

123 bp DNA ladder- suitable for determining the size of double-stranded DNA from 123 to 3,075 bp. Prepared from a plasmid containing 34 repeats of a 123-bp DNA fragment.

1 kb DNA ladder- suitable for sizing linear double-stranded DNA fragments from 500 bp to 12 kb. The bands of the ladder each contain from 1 to 12 repeats of a 1,018-bp DNA fragment.

ϕ X174 RF DNA/Hae III fragments- suitable for sizing linear double-stranded DNA from 72 to 1,353 bp. Prepared from purified ϕ x174 RF DNA digested to completion with Hae III.

M-MuLV reverse transcriptase- Moloney Murine reverse transcriptase is an RNA-dependent DNA polymerase that uses single-stranded RNA or DNA as a template to synthesize the complementary DNA strand.

mRNA- messenger ribonucleic acid

mRNP- messenger ribonucleoprotein particle (messenger RNA complexed with proteins)

NACS- Nucleic Acid Chromatography System

$(\text{NH}_4)_2\text{SO}_4$ - ammonium sulfate

nt- nucleotides

Nucleotides-

ATP- adenosine 5'-triphosphate

CTP- cytidine 5'-triphosphate

GTP- guanosine 5'-triphosphate

TTP- thymidine 5'-triphosphate

dATP- 2'-deoxyadenosine 5'-triphosphate

dCTP- 2'-deoxycytidine 5'-triphosphate

dGTP- 2'-deoxyguanosine 5'-triphosphate

dTTP- 2'-deoxythymidine 5'-triphosphate

dITP- 2'-deoxyinosine 5'-triphosphate

ddATP- 2', 3'-dideoxyadenosine 5'-triphosphate

ddCTP- 2', 3'-dideoxycytidine 5'-triphosphate

ddGTP- 2', 3'-dideoxyguanosine 5'-triphosphate

ddTTP- 2', 3'-dideoxythymidine 5'-triphosphate

PABP- poly(A) binding protein

PCR- polymerase chain reaction

PMSF- phenyl methyl sulfonyl fluoride, serine protease inhibitor

RNase- ribonuclease

RNasin- ribonuclease inhibitor, inhibits activity of RNase A-type enzymes.
 RNP-CS- ribonucleoprotein consensus sequence (K/R)G(F/Y)(G/A)FVX(F/Y)
 rRNA- ribosomal ribonucleic acid
 S phase- synthesis phase of cell cycle
 S1 nuclease- single strand specific endonuclease which hydrolyzes single-stranded RNA or DNA into 5' mononucleotides.
 SDS- sodium dodecyl sulfate
 Sequenasetm- modified (exonuclease deficient) T7 DNA polymerase.
 snRNP- small nuclear ribonucleoprotein particle
 SPC- serum protein coding
 SP6 sequencing primer- synthetic oligodeoxyribonucleotide for use in dideoxy sequencing in vectors containing the bacteriophage SP6 RNA polymerase promoter. Primer hybridizes to a conserved 20-base SP6 promoter sequence.
 SSC- (1X = 0.15M sodium chloride, 0.015M sodium citrate)
 Taq DNA polymerase- isolated from the thermophile bacteria Thermus aquaticus strain YT-1. It is a DNA dependent DNA polymerase with an optimum temperature of 80°C.
 TCA- trichloroacetic acid
 TEMED- N,N,N',N'-tetramethylethylenediamine
 TES- 10 mM Tris, pH 7.4 containing 1 mM EDTA and 1% SDS (wt/vol)
 Tris-HCl- [tris(hydroxymethyl)aminomethane hydrochloride]
 tRNA- transfer ribonucleic acid
 T4 polynucleotide kinase- catalyzes the transfer of the γ -phosphate of ATP to the 5'-hydroxyl terminus of DNA or RNA. It also has a 3' phosphatase activity.
 T4 RNA ligase- catalyzes the formation of phosphodiester bonds between the 5' phosphate of a donor molecule and the 3' hydroxyl of an acceptor molecule.
 T7 sequencing primer- synthetic oligodeoxyribonucleotide for use in dideoxy sequencing in vectors containing the bacteriophage T7 RNA polymerase promoter. The primer hybridizes to a conserved 20-base T7 promoter sequence.

T7DNAP- T7 DNA polymerase (Sequenasetm)

UV- ultraviolet

X-gal- (5-bromo-4-chloro-3-indolyl- β -D-galactoside)

X. laevis- Xenopus laevis, South African clawed toad

λ ZAP- insertion vector used for the construction of cDNA libraries. The cloning site is the C-terminal region of the lacZ gene. Recombinant phage may be recognized with blue/white color identification when plated on lac- hosts in the presence of IPTG and X-gal. DNA sequences cloned into the multiple cloning site of the vector may be expressed as fusion proteins under the control of the lac promoter. Libraries constructed in this vector can be screened with antibodies as well as nucleic acid probes. Strand-specific RNA transcripts can be generated from the T3 and T7 promoters flanking the multiple cloning site. Fragments cloned into the vector can be excised with helper phage and recircularized to generate subclones in the pBluescript SK- phagemid vector, eliminating time and labor intensive subcloning.

INTRODUCTION

The initiation of transcription is the first step along the complex pathway of gene expression. To understand why different gene products accumulate and decay at various rates, achieving final concentrations varying by orders of magnitude, one must consider each step involved in the biosynthesis of both mRNA and its functional protein. It is imperative not only to quantify the relative contributions of synthesis and degradation but also to identify the structural features and components involved in the degradation of different gene products.

Half-lives for eukaryotic mRNAs range from a few minutes for highly regulated gene products such as oncogenes and rate-limiting enzymes to hundreds of hours for more stable species. The average half-time of turnover for mRNA in eukaryotic cells is 10-20 hours. This appears to be a weighted average of the half-times of at least two kinetic populations, one relatively unstable, and one stable (Singer and Penman, 1973; Moore et al., 1980). In contrast, the average half-life for protein is 48-72 hours (Schimke, 1973).

I. ESTROGEN and XENOPUS ALBUMIN: A MODEL for mRNA STABILITY

This dissertation focuses on the role of mRNA stability in gene expression in Xenopus laevis, and the elucidation of the biochemical mechanisms regulating albumin mRNA turnover.

Albumin is an abundant, highly conserved protein of hepatic origin found in the sera of vertebrate animals. The serum of the frog Xenopus laevis contains two albumins of molecular weight 74,000 and 68,000 daltons (Bisbee et al., 1977); in contrast, most higher vertebrates possess only the lower

molecular weight species. Westley and Weber (1982) proposed that the 6,000 dalton difference in molecular weights between the two Xenopus albumins is primarily due to glycosylation of the 74 KDa protein and to a lesser degree due to differences in primary translation products. Schoenberg (1981) has shown that in vitro translation of hybrid selected albumin mRNA resulted in only one protein band of molecular weight approximately 72,000 daltons. In Xenopus, albumin is encoded by at least two genes termed 74 KDa and 68 KDa (May et al., 1983; Westley et al., 1981), or class I and II (Schoenberg, 1981).

One hormone that influences mRNA turnover is estrogen. In Xenopus, estrogen stimulates the production of the yolk precursor protein vitellogenin (Wallace and Jared, 1968; Follett et al., 1968; Whitliff and Kenney, 1968). Estrogen does so both by increasing the rate of transcription and by stabilizing the mRNA (Brock and Shapiro, 1983). The half-life of vitellogenin mRNA in estrogen-treated cells is >500 hours compared with 16 hours for the same mRNA in untreated cells. In contrast to this induction phenomenon, serum albumin mRNA levels in total RNA drop to 30% of control values by 12 hours after hormone administration. Albumin mRNA levels remain suppressed throughout the complete vitellogenic response to a single injection of estradiol (Riegel et al., 1986).

In initial studies, estrogen was found to have little effect on the rate of albumin transcription in isolated liver nuclei (Riegel et al., 1986). Therefore, it was proposed that the suppression of albumin mRNA is the result of a post-transcriptional mechanism (Riegel et al., 1986). However, the nuclear estrogen receptor of Xenopus liver appears to mediate the effects of estrogen on albumin degradation. Administration of 4-hydroxytamoxifen, an

estrogen receptor antagonist, blocks the estrogen-dependent degradation (Riegel *et al.*, 1987a,b).

This dissertation addresses four major topics: 1) determination of the location in the cell where the albumin degradation takes place; 2) identification of structural elements of an individual or class of mRNA(s) that may be recognized by the degradative component of the system; 3) determination of the selectivity, if any, of the degradative process, and 4) development of an *in vitro* cell-free assay system to characterize factors that modulate the interaction of the nucleolytic enzymes with mRNAs.

Regulated mRNA turnover is important to cell function. The steady-state abundance of a specific mRNA may be regulated by controlling its rate of synthesis and/or its rate of degradation (Rodgers *et al.*, 1985). While the mechanisms involved in mRNA synthesis have been investigated extensively, the pathways for the regulation of mRNA degradation remain poorly defined, particularly in eukaryotes. Studies on a wide range of model systems have demonstrated the importance of mRNA turnover in affecting gene expression. These studies have revealed that mRNAs, as a population, and sometimes individually, display a wide range of stabilities (Carneiro and Schibler, 1984; Carrera *et al.*, 1984). Individual mRNA species show characteristic rates of degradation that may differ greatly from the norm, but these rates are not fixed and may change in responsive tissues after hormone exposure (Guyette *et al.*, 1979; Brock and Shapiro, 1983; Schoenberg *et al.*, 1989; Pastori *et al.*, 1990), or exposure to other agents that may alter the growth state of the cell (Heintz *et al.*, 1983; Profous-Juchelka *et al.*, 1983; Mangiarotti *et al.*, 1983; Groudine and Casimir, 1984; Bandyopadhyay *et al.*, 1987; Kwong and Frankel, 1987; Wilson and Treisman, 1988).

Several ubiquitous mRNAs, encoded by so-called "housekeeping" genes, undergo changes in their turnover rates in response to changes in the cell cycle. Post-transcriptional events have been shown to regulate such essential mRNAs as those encoding thymidine kinase, dihydrofolate reductase, thymidylate synthetase and histones (Kaufman and Sharp, 1983; Carneiro and Schibler, 1984; Friedman *et al.*, 1984; Powell *et al.*, 1984; Krowczynska *et al.*, 1985). Viruses affect mRNA turnover in infected cells as described for herpes simplex virus (Kwong and Frankel, 1987). Another extrinsic molecule that regulates the turnover of mRNA is glucose which helps to stabilize insulin mRNA in pancreatic cells (Ross, 1989). Little is known about the mechanism through which extrinsic species regulate mRNA stability, but the process likely involves factors that interact with mRNA structural elements to account for the specificity and reversibility of mRNA stabilization (Brawerman 1987; 1989).

There is an advantage to cellular regulation of mRNA degradation. Even after synthesis of a particular mRNA has ceased, protein will be made from remaining mRNA transcripts. Increased rate of degradation of transcripts ensures a faster end to production of the regulated protein, and the rapid freeing-up of protein synthesis precursors and translational machinery. An example of this strategy occurs during differentiation of unspecialized bone-marrow cells into red blood cells (Aviv *et al.*, 1976). Synthesis of globin mRNA increases as total cellular RNA declines during maturation of reticulocytes, although the rate of transcription for the globin mRNA does not exceed 0.05% of the total (Krowczynska *et al.*, 1985; Ganguly and Skoultschi, 1985). Globin mRNA ultimately comprises 90% of total cellular mRNA, as a result of selective degradation of nonglobin mRNA and protein in mature red

blood cells (Volloch and Housman, 1981; Krowczynska et al., 1985).

Steroid hormones alter gene expression by regulating the rate of transcription of hormonally responsive genes (Chan et al., 1978). The central feature of the model for estrogen action is that estradiol evokes changes in the synthesis of specific proteins due to alterations in the steady-state concentrations of their cognate mRNAs. These events typically are mediated through a class of intracellular proteins which bind to DNA and alter the rate of transcription of specific genes (Ptashne, 1988). Selective transcriptional activation by steroid involves interaction of the steroid-steroid receptor complex with a specific nucleotide sequence in the DNA termed the steroid responsive element (reviewed by Evans and Hollenberg, 1988).

In addition to regulating gene expression at the level of transcription, hormones might alter the stability of a given mRNA by acting at any of the many steps in the pathway of RNA metabolism and subsequent translation. Beginning at transcription initiation, the selection of a different initiation site might result in the loss or introduction of stabilizing or destabilizing sequences at the 5' terminus of the message (Hagenbuchle et al., 1981; Grez et al., 1981; Heberlein et al., 1985; Piechaczyk et al., 1985; Shaw et al., 1985; Funzio et al., 1986). Hormone might also alter the splicing of the message, such that sequences affecting stabilization might be differentially expressed in the mature transcript (Green, 1986; Brawerman, 1987). Hormone might alter the rate of transport of mRNAs into the cytoplasm where steady-state levels are maintained (Young et al., 1986). Hormone could alter translation and post-translational processes in target tissues (Firestone et al., 1982, 1986; Haffar et al., 1987). There is also the possibility for differential utilization of polyadenylation

signals during cell growth and differentiation thereby introducing or removing determinants of mRNA stability (Early et al., 1980; Galli et al., 1988). Selective shortening of the 3' poly(A) tail by hormone treatment could also regulate mRNA stability (Mercer and Wake, 1985). There are reports of post-transcriptional "editing" of mRNAs in which the primary nucleotide sequence within the message is altered after transcription (Feagin et al., 1988; Shaw et al., 1988). Finally, hormones could affect formation of RNA-protein complexes and thereby alter RNA stability. Alterations in any of these processes could lead to altered regulation of gene expression.

This dissertation addresses whether estrogen might act at any of these steps to alter albumin mRNA stability. Determination of the compartments of the liver cell (nucleus, cytoplasm) in which estrogen-induced disappearance of albumin occurs will narrow the possibilities. I have also determined whether estrogen induces any covalent changes in albumin mRNA that might differentiate this message for destabilization.

Estrogen-induced mRNAs decay faster after hormone withdrawal than in the presence of estrogen (Palmiter and Carey, 1974; Cox, 1977; Hynes et al., 1979; Wiskocil et al., 1980; Brock and Shapiro, 1983) suggesting that estrogen might increase the rate of degradation of responsive mRNAs (Gordon et al., 1988; Palmiter and Carey, 1974; Hynes et al., 1979). The stability of a single mRNA species may be positively or negatively regulated by different compounds. For instance, dexamethasone has been shown to destabilize type I procollagen mRNA while the same mRNA is stabilized by transforming growth factor- β (Raghow et al., 1986).

II. mRNA STRUCTURE and STABILITY

The stability of mRNA may be controlled by the structure of the mRNA itself. Messenger RNAs vary in size but have basically similar organization composed of a 5' noncoding segment, a coding segment, a 3' noncoding segment and in practically all mRNAs a 3' poly(A) tail of approximately 100-300 nucleotides. Each of the four segments of a mRNA, including the three untranslated regions, can affect mRNA turnover.

A. 5' Noncoding Region

The 5' terminal noncoding regions of mRNAs have been shown to play an important role in mRNA stability. Removal of the 5' terminal cap structure from reovirus mRNAs was shown to cause an increased rate of mRNA degradation (Brawerman, 1987). Through deletion analysis, 5' leader segments of transcripts from *E. coli omp A* (Belasco et al., 1986), bacteriophage T4 gene 32 (Gorski et al., 1985), and human histone H3 (Morris et al., 1986) have also been shown to be involved in the decay process. These segments contain the ribosomal binding site. A 5' terminal 147 nucleotide segment of the *omp A* gene contained stability determinants which could be fused to less stable transcripts conferring greater stability (Belasco et al., 1986). A hybrid transcript with a termination codon placed at the end of the 5' *omp A* segment was not stabilized suggesting that mRNA stabilization involves ribosome interaction. A 40 nucleotide segment located near the translation initiator methionine in bacteriophage T4 transcripts has been shown to be important in stability (Gorski et al., 1985). Interestingly, the gene 32 transcript and hybrid T4-gene 32 transcripts were stable only in T4-infected cells, suggesting that stabilization involves interaction of the leader sequence with

a factor produced specifically in infected cells. The 5' leader sequence is also involved in the regulation of human histone H3 mRNA. Histone mRNAs are unstable except in S-phase cells during active DNA synthesis. A chimaeric β -globin-histone mRNA containing the 5' histone leader sequence was destabilized when DNA synthesis was blocked (Morris *et al.*, 1986). However, replacement of the 5' leader sequence with hsp 70 mRNA allowed histone mRNA to remain stable in the absence of DNA synthesis (Morris *et al.*, 1986). These findings show that some transcripts contain discrete structural determinants in the 5' region that can influence the decay rate of mRNA segments derived from other genes.

B. Coding Region

The influence of the coding region on mRNA stability has also been demonstrated. The best characterized example of the role of the coding region in mRNA stability is that of tubulin. In most animal cells, the level of tubulin expression is established by the intracellular concentration of tubulin monomers which modulate the stability of tubulin mRNAs through a post-transcriptional mechanism (Yen *et al.*, 1988). The sequences that are necessary and sufficient to specify regulated stability of β -tubulin mRNA have been shown by DNA transfection experiments (Yen *et al.*, 1988) to lie within the first 13 translated nucleotides which encode the amino acids Met-Arg-Glu-Ile. It is postulated that when this peptide protrudes from the ribosome and is recognized by free tubulin subunits, the mRNA is degraded by a ribosome-bound nuclease (Yen *et al.*, 1988). Other examples of the importance of coding regions include exon 1 of c-myc and in histone H4 mRNA (Marzluff and Pandey, 1988). In B-cell neoplasms, the rate of turnover of the truncated c-myc mRNA

is reduced compared to the normal cellular counterpart. Since in neoplastic cells, the juxtaposition of *c-myc* and immunoglobulin heavy chain genes often results in the removal of the leader and exon 1, it has been suggested that coding sequences may play an important role in mRNA stability (Piechaczyk *et al.*, 1985; Rabbitts *et al.*, 1985).

The length of the coding region may also be a determinant of mRNA stability. Histone genes were mutated so that when they were inserted into cells and transcribed, mRNAs of differing lengths resulted. The half-lives of the longer mRNAs were found to be at least twice as long as normal histone gene transcripts. The mutated mRNAs with repositioned stop signals formed abnormally configured polyribosomes that were unable to synthesize normal histone protein (Marzluff and Pandey, 1988). The structures of the coding segment, in this case its size, influenced the turnover of the mRNA.

C. 3' Noncoding Region

Sequences at the 3' end of mRNAs have also been shown to be involved in the decay process. A 3' AU-rich sequence is present in a variety of mRNAs from transiently expressed genes, such as *c-myc*, *c-fos* and γ -interferon. These mRNAs are normally very unstable, a feature that permits their rapid disappearance after induction. The *v-fos* mRNA is much more stable than its cellular homologue *c-fos* (Meijlink *et al.*, 1985). The viral and cellular *fos* oncogenes are homologues except for a unique 67 nucleotide sequence in the 3' untranslated region of *c-fos* message (Meijlink *et al.*, 1985; Treisman, 1985). When this segment is ligated to the 5' portion of *v-fos* the stability of the chimaeric mRNA declined to less than a half, suggesting that this sequence is involved in mRNA turnover. Furthermore, when the 67 nucleotide sequence was

deleted from the 3' untranslated region, the resulting c-fos transcript became much more stable. A fusion of this 3' sequence of c-fos mRNA to the 5' portion of β -globin mRNA rendered the globin transcript more susceptible to degradation ($t_{1/2}$ from 7 hr to 30 min). Shaw and Kamen (1986) found a highly conserved AU-rich sequence (50-60 nucleotides in length) in the 3' noncoding region of the human lymphokine GM-CSF mRNA important in regulating its stability. They positioned this sequence into the 3' untranslated region of the globin gene. The chimaeric globin-GM-CSF transcripts were degraded at rates 10-30 fold greater than normal globin transcripts.

How the AU-rich sequences promote mRNA decay is not known. Possibly the AU-rich sequence could be cleaved by a specific endonuclease, which would leave the mRNA susceptible to 3' exonucleolytic attack (Brawerman, 1987). This hypothesis is supported by the detection in maturing reticulocytes of truncated β -globin mRNA chains generated by cleavages at AU sites in the 3' noncoding region (Albrecht et al., 1984). A cytosolic protein that binds to RNA molecules containing reiterations of the AUUUA structural element has been identified (Malter, 1989), and it is believed that the formation of the protein-AUUUA RNA complex may target susceptible mRNAs for rapid cytoplasmic degradation.

D. Role of Poly(A) Tract in mRNA Degradation

Most mRNAs in animal cells possess a 3'-terminal tract of 100-300 adenosine residues (poly(A) tail). This poly(A) tail is added to the mRNA in the nucleus after transcription. Addition of the poly(A) tail requires a specific endonucleolytic cleavage of the nascent pre-mRNA and later addition of adenylate residues. A highly conserved AAUAAA sequence (Manley et al.,

1985; Zarkower and Wickens, 1987), located within 30 nucleotides of the poly(A) tail of virtually all eukaryotic mRNAs is also required. The poly(A) tail may be lengthened or shortened after the polyadenylated transcript is transported into the cytoplasm (reviewed by Lewin, 1980).

In vitro, mRNA-specific poly(A) addition assay systems have been described. Poly(A) addition requires at least two components; a poly(A) polymerase and a specificity factor that selects only RNAs containing AAUAAA to receive poly(A) (Christofori and Keller, 1988; 1989; Takagaki et al., 1988). Wigley et al. (1990) found that a substrate that contained only 11 nucleotides, of which the first six were AAUAAA, underwent AAUAAA-specific polyadenylation. Removal of a single nucleotide from either end of this RNA abolished the reaction.

The 3' poly(A) tract is believed to have multiple functions both in the nucleus and cytoplasm of cells. It might play a role in the processing and transport of mRNA, it affects the translational efficiency of mRNAs, and it influences mRNA turnover.

Relationships between poly(A) metabolism and mRNA degradation has been debated since the discovery of poly(A) tails over 20 years ago (Edmonds and Caramela, 1969). A correlation between poly(A) length and mRNA stability has been observed in some (Wilson et al., 1978; Zeevi et al., 1982; Paek and Axel, 1987; Wilson and Treisman, 1988) but not all mRNAs (Deshpande et al., 1979; Muschel et al., 1986). Deadenylated globin mRNA is unstable in microinjected frog oocytes (Marbaix et al., 1975) and deadenylated mRNAs can be stabilized by polyadenylation (Graves et al., 1987; Huez et al., 1981). Stabilization and/or accumulation of several mRNAs is accompanied by elongation of their poly(A) tracts. Stabilization of human growth hormone mRNA by glucocorticoids

correlates with lengthening of its poly(A) tract (Paek and Axel, 1987). When rats are subjected to osmotic stress, vasopressin mRNA accumulates to higher levels and its poly(A) tract is lengthened by 150 residues (Zingg *et al.*, 1988; Robinson *et al.*, 1988). Similar links between poly(A) length and changes in the stability and translatability of maternal mRNAs has been seen following fertilization and during early embryogenesis (Richter, 1987). The resulting deadenylated RNA then forms a labile substrate for further degradation.

Messenger RNA degradation can follow a sequential pathway starting with poly(A) removal. This is evident in the degradation of c-myc mRNA in exponentially growing human myeloid cells (Swartwout and Kinniburgh, 1989) and in c-fos mRNA decay in HeLa cells (Wilson and Treisman, 1988). AU-rich sequences upstream of the poly(A) tail can act to direct rapid shortening (Brewer and Ross, 1988).

The poly(A) tract alone may not be responsible for mRNA stability. Poly(A)-binding proteins (PABPs) exist and bind with high specificity to the poly(A) region. This will be discussed under trans factors and mRNA stability.

Influence of Secondary Structure on mRNA Degradation:

Terminal secondary structures in mRNAs could possibly influence the rate of mRNA digestion by exonucleases. Examples include the rho-independent transcription termination signals which contain putative hairpin structures that protect against 3' exonuclease activity (Wong and Chang, 1986). Fusion of the terminator sequence to the 3' end of other genes confers stability to the resultant transcripts (Wong and Chang, 1986). A similar sequence has been

identified in ϕ X174 transcripts (Hayashi and Hayashi, 1985), and mutations that reduce the sequences capability of forming stem-loop structures also reduce its stabilizing effect. Hairpin structures may also impart stability to selected regions of mRNAs encoding DNA primase. It is postulated that the exposed 3' terminus of the primase region is degraded processively in a 3' to 5' direction until the stem-loop structure is reached (Burton et al., 1983). Some of the histone mRNAs contain a sequence with the potential to form a stem-loop structure at the 3' end possibly making this region resistant to 3' exonucleases (Georgiev and Birnstiel, 1985). Regulation of histone mRNA degradation requires optimum positioning of this highly conserved 3' stem-loop structure from the translation termination codon. The mRNA encoding the transferrin receptor is destabilized by high iron levels, and stabilized when iron is scarce. The stabilization of transferrin receptor mRNA, in the absence of iron, requires the 3'-untranslated region of the mRNA which has the potential to form a series of stem and loop structures. The stability of mRNAs containing putative secondary structures may be mediated by proteins which could bind to these structures making the protein-mRNA complex more (histone) or less (transferrin receptor) susceptible to cleavage by degradative machinery of the cell (Peltz and Ross, 1987).

Coordinate changes in rates of translation and altered mRNA secondary structure in higher eukaryotes are exemplified by studies on eukaryotic initiation factor 4F (eIF-4F), an ATP-dependent, unwinding enzyme capable of catalyzing the melting of mRNA to a state of increased nuclease sensitivity. It is reasoned that eIF-4F functions to decrease intramolecular RNA duplexes which may inhibit ribosome binding and consequently disrupt protein synthesis (Kozak, 1980). Conversely, ribosomes that initiate and terminate translation

normally, may prevent the formation of a structure that destabilizes the mRNA, or allow the formation of a structure that confers stability to the mRNA.

Although it is clear that primary and secondary sequences of mRNAs influence their stability, the mechanisms by which stability is modulated are presently unknown. Alterations in the degradation of specific mRNAs could be achieved by modulating the enzymatic activity, or the specificity of nucleases involved in the initial stages of degradation or by altering the accessibility of susceptible sites in the mRNA.

III. Trans FACTORS and mRNA STABILITY:

Even though the stability of a particular mRNA may be a result of multiple structural elements, interaction of the message with other factors is required, to effect degradation in vivo. Messenger RNA destruction results from the action of cytoplasmic ribonucleases (RNases) or other mRNA binding molecules.

Most cells are believed to contain several ribonucleases. The sequence of events leading to mRNA degradation is not well understood. Bacterial ribonucleases have been shown to degrade RNA sequentially from the 3' to 5' end. These include RNase II, D, T, and RW and polynucleotide phosphorylase (Donovan and Kushner, 1986; Deutscher, 1988). Prokaryotic endonucleases have also been described and include RNase I, III, P, E, and H (Brawerman, 1987; 1989; Deutscher, 1988). It is not clear whether a single ribonuclease degrades only one class of RNA or all classes of RNAs. Little is known about eukaryotic ribonucleases.

Modulation of ribonuclease activity by endogenous inhibitors has been shown in several systems (Swida et al., 1981; McGregor et al., 1981; Nilsen et

al., 1982; Jacobsen et al., 1983; Penttila and Maenpaa, 1985). Total ribonucleolytic activity may be determined in part by the ratio of ribonuclease to ribonuclease inhibitor. The level of ribonuclease inhibitor often increases when cells are stimulated by hormones or mitogens. Estrogen treatment can alter the level of ribonuclease activity in the liver and the hormone specifically influences the stability of apo VLDL II mRNA (Wiskocil et al., 1980; Penttila and Maenpaa, 1985).

The possibility that ribosomes can protect mRNAs from degradation by cellular nucleases has also been examined. This idea was supported by early studies of the trp operon (Morse and Yanofsky, 1969). However, recent work indicates that protection by ribosomes may not be a significant factor in mRNA decay. The stability of lac Z transcripts that differ in their ability to initiate translation (differ in the number of ribosomes covering the coding region) were compared and no differences in decay rates were found (Stanssens et al., 1986). In other studies, it has been shown that the most stable portion of the E. coli omp A transcript (coding for the bacterial outer membrane protein) lies in the 5' noncoding region which supposedly is not covered by ribosomes, providing additional evidence against ribosome protection (Stanssens et al., 1986).

A. RNA Binding Proteins:

In eukaryotic cells, mRNA is found complexed with a variety of proteins. Nuclear complexes are termed heterogeneous ribonucleoprotein particles (hnRNPs) while cytoplasmic complexes are termed messenger ribonucleoprotein (mRNP) particles. Many investigators have shown that mRNPs are found both associated (or bound) with polyribosomes (being actively

translated), and in <80S sedimenting fractions (free or unbound) (Vincent *et al.*, 1981). The composition of the cytoplasmic mRNP complexes varies with the translational state of the mRNA as certain proteins present in polyribosomal mRNA-protein complexes are absent or reduced in amount in 40 to 80S complexes and in complexes formed in the absence of translation (Greenberg and Carroll III, 1985). Messenger ribonucleoprotein particles are dynamic structures with their proteins exchanging in the cytoplasm with a pool of similar proteins not bound to mRNA (Greenberg, 1981).

Structural or primary sequence features of proteins that participate in specific RNA-protein interactions are now being characterized. The observation of an RNP consensus octamer (K/RGF/YG/AFVXF/Y) in several eukaryotic proteins associated with RNA was the first sign of an amino acid sequence common among some RNA binding proteins (Bandziulis *et al.*, 1989). These proteins frequently contain several similar RNP-CS type RNA-binding domains and at least one auxiliary domain unique to each type of protein. The auxiliary domain is believed to function in protein-protein interactions. Among the most studied RNA-binding proteins are the A1, C1 and E proteins found in hnRNP, the U1 protein found in snRNP, nucleolin pre-mRNP and poly(A) binding protein (PABP). These proteins have been implicated in pre-mRNA splicing, polyadenylation, mRNA stability, and as accessory factors for protein synthesis/translational regulation.

Poly(A) binding protein is believed to play a role in mRNA stability. PABP binds 100 times more tightly to the poly(A) tail than to other mRNA segments and has the ability to organize poly(A) into repeating units occupying about 25 residues (Richter and Evers, 1984; Richter and Smith, 1984; Swiderski and Richter, 1988). The decay rate of β -globin mRNA was compared in

ribosomal salt extracts containing PABP or the same extracts depleted of PABP by chromatography through a poly(A)-Sepharose column (Brewer and Ross, 1988). Globin mRNA was degraded seven times faster in reactions deficient in PABP than with PABP, and degradation followed a sequential pathway in which poly(A) removal preceded further mRNA degradation (Brewer and Ross, 1988). When purified PABP was added back to the salt extract, the mRNA was stabilized, supporting the idea that a poly(A)-PABP complex is necessary to maintain the half-life of mRNAs, at least in vitro. Therefore, PABP appears to stabilize mRNA by protecting the poly(A) segment against attack by ribonucleases. It is unknown how PABP protects different poly(A) mRNAs to various extents.

B. Effect of Protein Synthesis on mRNA Degradation

There are several systems in which mRNA stability has been shown to be altered by changes in protein synthesis and ribosome loading. Tubulin mRNA degradation in the presence of excess tubulin monomers requires the mRNA to be in a polyribosome, and is enhanced in the presence of cycloheximide (Pachter et al., 1987; Yen et al., 1988 a,b). For degradation of histone mRNA, not only must the RNA be translated, but the ribosome must translocate to within 300 nucleotides of a stem and loop structure on the message, a probable site of initial cleavage by a ribosome-associated nuclease (Graves et al., 1987; Pandey and Marzluff, 1987; Ross et al., 1987). The regulation of casein mRNA stability has been shown to be dependent on the synthesis of a putative labile protein (Poyet et al., 1989) similar to the regulation of c-fos mRNA stability in human monocyte differentiation (Weber et al., 1989). Destabilization of hsp 70 mRNA upon recovery from heat shock has been linked to continuing translation (Banerji et al., 1986; DiDomenico et al., 1982). Termination of

protein synthesis has been shown to destabilize triosephosphate isomerase mRNA (Daar and Maquat, 1988) and yeast URA3 and PGK1 mRNAs (Hoekema *et al.*, 1987; Losson and Lacroute, 1979). These investigators suggest that the ability of a ribosome to traverse the mRNA and to either impart a specific structure to the mRNP particle, or to approach a specific 3' site on the mRNA may be important in controlling mRNA degradation (Daar and Maquat, 1988).

Little is known of the role played by protein synthesis and ribosome loading in hormone mediated regulation of mRNA stability. Protein synthesis is required for ecdysone-inducible middle and late puffs in *Drosophila* polytene chromosomes (Ashburner *et al.*, 1973). Brock and Shapiro (1983) have shown that inhibition of protein synthesis with cycloheximide did not prevent the stabilization of vitellogenin mRNA in the presence of estrogen, or its destabilization upon removal of estrogen. Using protein synthesis inhibitors specific for the initiation (MDMP) and elongation (cycloheximide) steps, they demonstrated that a high density of ribosomes on vitellogenin mRNA is required for the estrogen-mediated stabilization of vitellogenin mRNA (Blume and Shapiro, 1990). Studies in other laboratories indicate that a decrease in the density of ribosomes on an mRNA accelerates its degradation (Daar and Maquat, 1988; Hoekema *et al.*, 1987; Losson and Lacroute, 1979).

In addition to proteins affecting RNA stability, RNAs themselves have been shown to contain catalytic activity. Although there are four types of RNA-catalyzed reactions, the underlying chemistry is similar: removal of a proton from a properly positioned oxygen triggers an attack by that oxygen on the electrophilic phosphorous in the phosphodiester bond that is broken. The role catalytic RNAs play in RNA turnover is unknown.

IV. In vitro CELL-FREE SYSTEMS to STUDY RNA DEGRADATION

In vitro cell-free systems have been developed to address the mechanisms of mRNA stability (Ross et al., 1986; Peltz et al., 1987; Brewer and Ross, 1989; Pei and Calame, 1988; Sunitha and Slobin, 1987). These systems accurately reflect mRNA turnover processes in whole cells as the rank order of decay of various mRNAs in vitro reflects that seen in intact cells. It is therefore possible to identify structural features of the mRNA, and the trans-acting factors such as proteins or metal ions needed for mRNA degradation using these assays. Messenger RNA turnover is studied in test-tube mixtures containing RNA complexed as polyribosomes or mRNP particles. Cell-free systems that degrade mRNA have been prepared from reticulocytes and other hematopoietic cells (Wreschner and Rechavi, 1988; Ross and Kobs, 1986). At least two ribonuclease activities have been described. One cleaves at segments with a general motif of U_nA (Beutler et al., 1988); the other is RNase L which is activated by enzymatic production of 2',5'-linked oligoadenylates (Wreschner and Rechavi, 1988). Ribonucleases have been found associated with polyribosomes (Ross et al., 1986; Bandyopadhyay et al., 1990) as well as mRNP particles (Sunitha and Slobin, 1987; Bandyopadhyay et al., 1990). The Ross group (Peltz et al., 1987; Brewer and Ross, 1989) has characterized the properties of the enzymatic activity that degrades histone mRNA using an in vitro system. However, to date, this group has not purified to homogeneity the ribonuclease(s).

Similar in vitro systems for the study of mRNA stability have been described by two other laboratories. Sunitha and Slobin (1987) constructed an in vitro system derived from stationary Friend erythroleukemia cells, consisting of 40-80S mRNPs. They found that some mRNAs, including ones

encoding for eukaryotic elongation factor Tu and PABP, were rapidly degraded, whereas most of the mRNAs present on mRNPs were stable. Finally, Pei and Calame (1988) demonstrated that the differential stability observed between normal, full-length c-myc mRNA and truncated c-myc mRNA in vivo can be reproduced in an in vitro system. Crude cell extracts prepared from plasmacytoma cells were lysed. Nuclei, mitochondria, and lysosomes were removed by centrifugation, and the resulting supernatant was incubated with ³²P-labelled mRNA. Chimaeric mRNA were constructed consisting of the first exon of c-myc fused to either immunoglobulin C heavy chain or glyceraldehyde-3-phosphate dehydrogenase. Both transcripts were not rapidly degraded, demonstrating that c-myc exon 1 alone is not sufficient to target mRNAs for rapid degradation (Pei and Calame, 1988).

The advantages of using in vitro systems to study mRNA degradation are many. Transcriptional inhibitors which can interfere at other steps in gene regulation are not needed. Additionally, short-lived mRNA decay products that are difficult to detect in cells may be observed in vitro where reaction rates are considerably slower. Finally, it is possible to analyze the effects of cellular components that might regulate mRNA stability and to purify biochemically the factors involved. It is only with in vitro systems that one can fully begin to understand the regulation of RNA stability.

A. Conditions for Degradative Activity in Cell-Free Systems

Requirements for degradative activity have been studied using in vitro assay systems (Ross et al., 1987; Sunitha and Slobin, 1987; Pei and Calame, 1988). It is not clear that degradation of exogenous mRNA in vitro requires ATP, but mRNA degradation was initially reconstituted in the presence of an ATP-generating system (Ross et al., 1987). Most nucleases do not require ATP

for hydrolysis of covalent bonds in their substrates, but it would not be surprising if regulated degradation in vivo requires energy. Degradation requires disruption of RNA-protein structures, an energy-dependent process. ATP has been suggested to be required to activate a latent ribonuclease or to promote other steps involved in hormone-dependent stabilization or destabilization (Shapiro et al., 1987; Ross, 1988). In addition to a possible energy requirement, the need for divalent cations for activity has been seen in some systems (Ross et al., 1987; Pei and Calame, 1988) but not in others (Sunitha and Slobin, 1987). Furthermore, temperature and pH has been shown to influence ribonuclease activity (Ross et al., 1987; Pei and Calame, 1988).

B. Degradative Products

Although alterations in stability have been shown to be important in determining the abundance of an increasing number of mRNAs, data on the degradative process itself are not available in most cases. Degradative products of histone H4, β -globin, and apo VLDLII mRNAs have been studied. The first detectable decay product of histone H4 mRNA lacks 5 to 15 nucleotides from the 3' terminus (Ross et al., 1987). Histone mRNAs in one sense are atypical because of their lack of a poly(A) tract at the 3' terminus. Degradation products for rabbit β -globin have been characterized. Its degradation intermediates result from endonucleolytic cleavage in regions rich in AU (Albrecht et al., 1984). Cochrane and Deely (1989) characterized the in vivo degradative intermediates of apo VLDLII mRNA following estrogen withdrawal, and found discrete intermediates consisting of fragments with intact 5' ends but partially degraded 3' ends. The end points of the fragments mapped in or within four nucleotides of the tetranucleotide GAUG. Based on secondary structures predicted by folding programs, the end points

appear to be preferentially located at internal bulges in stem and loop structures. Binder et al. (1989) also identified specific degradation intermediates of apo II mRNA. The typical cleavage site was a 5'-AAU-3' or 5'-UAA-3' trinucleotide in an accessible single-stranded loop domain. They proposed that endonucleolytic cleavage within the 3'-noncoding region would remove the poly(A) tail from apo VLDLII mRNA and make the resulting fragments susceptible to exonucleolytic digestion.

Secondary structures in the translated portion of the molecule are likely to be restricted to a fraction of the mRNA population that is not loaded with ribosomes, such as free mRNPs or RNA associated with ribosomal subunits and monosomes. In these systems, it is not clear whether degradation can begin by cleavage at any one of the end points detected or whether the spectrum of intermediates observed is generated by a processive mechanism. The identification of common features of primary sequence and secondary structure need to be correlated with the end points of specific degradative intermediates. Structural features can then be imparted on chimaeric mRNAs to test their effect and thereby distinguish these possibilities.

In this thesis an in vitro system was developed to study estrogen-regulated albumin mRNA destabilization. This is the first such in vitro assay system to study hormonally-regulated RNA stability. It is hoped that the development of this system will allow for the biochemical purification of factors controlling RNA stability.

MATERIALS & METHODS

Experimental Animals

Laboratory bred male Xenopus laevis were obtained from Nasco, Inc. (Fort Atkinson, WI) and kept in Plexiglas aquaria at 20°C with a 12-hr light, 12-hr dark cycle. Frogs were fed frog brittle twice weekly. Injections of 1 mg of estradiol were performed in 0.1 ml 5% (v/v) dimethyl sulfoxide in propylene glycol in the dorsal lymph sac. Control animals received injection vehicle 24 hr prior to sacrifice. Before sacrifice, animals were anesthetized by immersion in a solution of tricaine methanesulfonate. Livers were perfused of blood with sterile 1X SSC (1X SSC = 0.15 M NaCl, 0.015 M sodium citrate), rinsed in cold 1X SSC and used immediately for the isolation of RNA.

Enzymes, Isotopes, and Reagents

Restriction enzymes and ultrapure chemicals were purchased from Bethesda Research Labs, Inc. (Gaithersburg, MD). Modified T7 DNA polymerase (Sequenase™) was purchased from United States Biochemical Corp. (Cleveland, OH). AMV reverse transcriptase (Berger et al., 1983) was purchased from Seikagaku Ltd. (Miami, FL). T4 DNA polymerase, T4 polynucleotide kinase and calf intestinal phosphatase are obtained from Boehringer-Mannheim (Indianapolis, IN). SP6 polymerase and T7 polymerase are produced by Promega Biotech. (Madison, WI). All other reagents were the highest quality available from Sigma Chemical Co. (St. Louis, MO). Radiolabeled nucleotide and deoxynucleotide triphosphates were obtained from New England Nuclear (Boston, MA).

Isolation of cDNA Clones for Xenopus 68 and 74 KDa Albumins

Albumin clones were isolated by screening a λ ZAP library prepared from male Xenopus laevis poly(A) RNA using albumin cDNA clone pXla14 as previously described (Schoenberg, 1981). The DNA inserts were recovered from the λ ZAP phage vector by an efficient in vivo excision process carried out by filamentous phage gene II protein (Short et al., 1988). This excision process resulted in a mixed culture containing the λ ZAP phage, filamentous helper phage, E. coli cells, and the excised Bluescript phagemid packaged as a filamentous phage particle. This mixed culture was heated at 68°C for 20 min which inactivates the λ ZAP phage particles and E. coli cells. The remaining culture contained the packaged Bluescript phagemid and the packaged helper phage. The final step of the excision process separated the Bluescript phagemid from the helper phage by infecting a fresh culture of E. coli with the phage mixture and plating on ampicillin plates. Only clones containing Bluescript phagemids survived the ampicillin selection thus completing the excision process.

Plasmids were isolated using the base-acid procedure of Birnboim and Doly (1979), followed by centrifugation in two-step CsCl-ethidium bromide gradients (Garger et al., 1983). Small-scale growth and isolation of plasmid DNA essentially follows procedures described by Holmes and Quigley (1981). This DNA was of sufficient quality for DNA sequencing. Small scale isolation of phage was performed according to Helms et al. (1985). Large scale isolation of phage follows the procedure outlined by Maniatis et al. (1985). Plaque screening utilized nitrocellulose filters (BA85, 0.45 μ m, Schleicher and Schuell) as the supporting medium in a procedure modified from that of Benton and Davis (1977; Hedrick et al., 1984).

DNA Sequence Analysis

The original cDNA insert from pXla14 was subcloned in opposite orientations into pGEM3Z for the preparation of overlapping deletion fragments with exonuclease III and mung bean nuclease (Henikoff, 1984). This was used to isolate additional clones for the 68 and 74 KDa albumins from a cDNA library of liver poly(A) RNA of a single male Xenopus prepared in λ ZAP. The cloned cDNAs were rescued with fl helper phage and maintained as plasmids in Bluescript. Double-stranded DNA sequencing was performed by a modification of the method of Chen & Seeburg (1985) using oligonucleotide primers complementary to the promoters of the cloning vectors (pBluescript SK- or pGEM3Z), modified T7 DNA polymerase (SequenaseTM) and [³⁵S]-dATP. To bridge gaps in the sequence, the same procedure was used with oligonucleotides derived from the preceding sequence. The sequence of terminal regions of pXla14 was determined by Maxam and Gilbert (1980) sequencing of end-labelled restriction fragments. The complete sequence of both strands was determined, compiled and analyzed with the GEL, SEQ, and PEP utilities of the Bionet resource. Additional analyses were performed with programs purchased from International Biotechnologies Inc. (New Haven, CT). A detailed description of the modified DNA sequencing reaction protocol is described in the Appendix.

Isolation of Total, Nuclear, and Cytoplasmic RNA Fractions

The procedure for the isolation of nuclear and cytoplasmic RNA was developed by Dr. Schoenberg and is described below. For isolation of total RNA, livers from individual frogs were homogenized with a polytron homogenizer in 4 M guanidine isothiocyanate in 50 mM sodium acetate, pH 5.5, containing 1 mM EDTA. For isolation of nuclear and cytoplasmic RNA, livers from two

frogs were homogenized in 25 ml of ice-cold 5% (wt/vol) citric acid, 1 M sucrose in a motor-driven teflon-glass homogenizer. The homogenate was filtered through sterile Nytex mesh (27621, Tetko) and centrifuged for 10 min at 6000 x g in a swinging bucket rotor. Solid guanidine isothiocyanate (0.47 gm/ml) was quickly dissolved in the post-nuclear supernatant (cytoplasmic RNA fraction) followed by 3 M sodium acetate, pH 5.5, to a concentration of 50 mM. An equal volume of cold ethanol was added to the cytoplasmic RNA fraction and this was stored at -20°C for 30 min (during which time the nuclear RNA fraction was isolated). The precipitated cytoplasmic RNA fraction was collected by centrifugation at 10,000 x g for 30 min at 2°C and the pellet was dissolved in 10 ml of 4 M guanidine isothiocyanate in 50 mM sodium acetate, pH 5.5, 1 mM EDTA. The final volume was adjusted to 15 ml with this solution.

The pellet of crude nuclei was suspended with the aid of a Dounce (B pestle) homogenizer in 6.5 ml of 1.5% citric acid, 1 M sucrose, and layered on an equal volume of 1.5% citric acid, 1.5 M sucrose. This was centrifuged at 6000 x g for 15 min. The nuclear pellet was resuspended with the Dounce (B pestle) in 7 ml of 1.5% citric acid, 1 M sucrose and a portion was removed, stained with trypan blue and counted on a hemocytometer. The nuclei were again centrifuged for 10 min at 6000 x g and the pellet was suspended in 4 M guanidine isothiocyanate, 50 mM sodium acetate, pH 5.5, containing 1 mM EDTA. To aid in the recovery of nuclear enriched RNA this material was homogenized vigorously with a Dounce homogenizer, first with the B pestle, followed with the A pestle. This step was necessary to shear the large amount of DNA present in the preparation.

Solid CsCl was added to each of the RNA fractions (1 gm/2.5 ml) and they were layered onto a 1.5 ml pad of 5.7 M CsCl in 0.1 M EDTA, pH 7.5. The

sealed tubes were centrifuged overnight at 100,000 x g in a 50 Ti rotor (Beckman Instruments) at 20°C. The RNA pellets were dissolved in 2 ml 10 mM Tris, pH 7.4, containing 1 mM EDTA, 1% (wt/vol) SDS (TES), and gently extracted with an equal volume of HCCl_3 :butanol (4:1). The aqueous phase was removed and the organic phase was reextracted with 1 ml of TES. The combined aqueous phases were extracted with an equal volume of buffer-saturated phenol to remove the last traces of contaminating melanin. The RNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate, pH 5.5 and 2.5 volumes of cold ethanol. This was stored at -20°C until further analysis.

Plasmid Clones

Cloned DNA inserts for hybridization to RNA blots were isolated from the albumin cDNA clones pXla14, pXla68, pXla74 (68 and 74 KDa albumin clones prepared from λ ZAP library) and the vitellogenin cDNA clone pXlvc10 by digestion with Eco RI and Hind III or Eco RI alone. pSPxv10/64, a subclone of the Eco RI-Hind III fragment of pXlvc10 was prepared by Dr. A. Riegel. The original vitellogenin clone (pXlvc10) was a gift from Prof. Walter Wahli (University of Lausanne, Switzerland). The actin cDNA insert was isolated by digestion of the tadpole-derived cardiac alpha-actin clone pSPAC9 with Eco RI and Bam HI. The latter clone was a gift from Drs. Tom Sargent and Igor Dawid (NIH). The probe used for RNase H analysis of albumin mRNA was a genomic subclone containing the last 3 exons of the 68 KDa (class II) albumin gene. A 350 bp Rsa I fragment from intron 14 was used to identify the primary albumin transcript in nuclear RNA preparations. The 3' untranslated albumin sequences were obtained from 74 and 68 KDa albumin clones isolated from a λ ZAP library prepared from male Xenopus laevis liver poly(A) RNA.

Electrophoresis and Northern Blot Analysis of RNA

Samples ranging from 1 to 10 μ g of RNA were glyoxalated to denature secondary-structure (McMasters and Carmichael, 1977), electrophoresed and blotted onto nylon membrane (Nytran, 0.45 μ m, Schleicher and Schuell, Keene, NH) by standard procedure (Thomas, 1980). Briefly, the RNA was precipitated in a microfuge tube and dissolved in 4 μ l of sterile water. Three μ l of deionized glyoxal, 2 μ l of 0.1 M sodium phosphate buffer and 8 μ l of dimethylsulfoxide was then added. The solution was heated at 50°C for one hr. At the end of one hr the RNA samples were removed from the water bath and cooled to room temperature. Four μ l of 5X loading buffer was added and the samples loaded onto an agarose gel. The gel apparatus was equipped with stirring bars in both buffer reservoirs and tubing for recirculation of the electrophoresis buffer (0.01 M sodium phosphate buffer, pH 7.0) with a peristaltic pump. Electrophoresis was performed at 100 volts. When the xylene cyanol tracking dye had entered the gel, stirring was commenced and buffer recirculated at a flow rate of 10 ml/min. Electrophoresis continued until the bromophenol blue was 1 cm from the end of the gel. The gel was removed from the boat and placed onto a blotting apparatus containing 10X SSC. Blotting was performed for 4-18 hr onto Nytran that was first treated with distilled water and then 10X SSC. The blot was then rinsed with 6X SSC and allowed to air dry for 30 min. The RNA was crosslinked to the membrane by exposure to ultraviolet light in a tissue culture hood for 2 min. The blot was prehybridized for 10 min at 65°C in Church buffer (Church and Gilbert, 1984) (0.5 M sodium phosphate, pH 7.0; 1 mM EDTA; 1% BSA; 7% SDS) containing 100 μ g/ml sheared, heat-denatured salmon sperm DNA. The prehybridization solution was removed and replaced with the same solution containing the

denatured probe. Concentrations of probe were chosen to achieve $10 \times C_{ot_{1/2}}$ in an 18 hr hybridization. The blots were hybridized overnight at 65°C.

Hybridization and washing conditions follow standard procedures (Minson and Darby, 1982; Gal *et al.*, 1983). The blot was washed twice for 15 min under each of the following conditions: 1) 10X SSC, 1% SDS, 25°C; 2) 1X SSC, 1% SDS, 37°C; 3) 0.1X SSC, 1% SDS, 65°C for well-matched hybrids; 3) 1X SSC, 1% SDS, 65°C for less matched hybrids. The blot was then dried and specific hybridization was detected by autoradiography on Kodak X-Omat AR XAR-5 film with Dupont Cronex Lightning Plus intensifying screens. The hybridized probe was removed from selected blots by heating for 15 min at 90°C in 0.1X SSC + 1% SDS. Blots could be re-probed. RNA size markers were obtained from Bethesda Research Laboratories. RNA for cytoplasmic dot blots was prepared essentially as described by White and Bancroft (1982) and modified by Papavasiliou *et al.* (1986). Vitellogenin, albumin and actin cDNA inserts were isolated from their respective plasmids and radiolabelled by priming with random hexamers (Feinberg and Vogelstein, 1983). Random hexamers were prepared by Mike Flora, Department of Pathology.

Electrophoresis of DNA was performed on alkaline agarose gels (Favaloro *et al.*, 1980), low melting agarose gels (Southern, 1979) and on agarose or polyacrylamide gels for restriction mapping (Helling *et al.*, 1974; Southern, 1979) or sequencing (Biggin *et al.*, 1983). Size markers (100 bp and 1 kb ladders, and restriction digests of pBR322 plasmids or lambda vectors) were purchased from Bethesda Research Laboratories.

Primer Extension Analysis of Albumin mRNA

Primer extension analysis was performed as described by Chepelinsky et al., (1987). Briefly, the primer ATGGTCTACATCTGT, which is complementary to the sequence from the end of exon 1 and the beginning of exon 2 of the 68 KDa albumin gene (Sweeney et al., 1987; Schorpp et al., 1988), was 5' end-labeled with γ -[32 P]ATP and T4 polynucleotide kinase. 10^6 dpm of primer was annealed to 15 μ g poly(A) RNA in 30 μ l of 50 mM Tris-HCl, pH 8.3, containing 100 mM KCl, 0.33 mM EDTA, 6 mM $MgCl_2$, 10 mM dithiothreitol by heating to 65°C for 10 min and then incubating at 42°C for 1 hr. After addition of 250 μ M dNTPs, 0.1 mg/ml actinomycin D, and 30 units of AMV reverse transcriptase, primer extension was performed by incubation at 42°C for 2 hr. The primer extension products were analyzed on a 10% acrylamide-urea sequencing gel.

Preparation of Liver Fragment Cultures

Livers from naive male Xenopus laevis were excised, cut into 5-10 mm cubes, allowed to adapt for 48 hrs and cultured at 22°C in 0.65X modified (Wangh and Schneider, 1982) Coon's medium (Ambesi-Impimbato et al., 1980) as described by Brock and Shapiro (1983). The media was supplemented with 20 mM HEPES buffer (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid), pH 7.4; glutamine (16 mg/ml); gentamycin (0.1 mg/ml); penicillin G (200 units/ml); streptomycin (0.2 mg/ml); bovine insulin (12.5 munits/ml), 10^{-8} M triiodothyronine and, as indicated, 10^{-6} M estradiol. To inhibit 95% protein synthesis, either 0.2 μ g/ml cycloheximide, 200 μ g/ml puromycin, or 20 μ g/ml MDMP was added to the medium. 10^{-6} M actinomycin D was added to inhibit 95% [3 H]uridine incorporation. Each 60 mm diameter plastic dish contained approximately 0.3-0.5 g liver in 5 ml culture medium. Cultures were covered

with aluminum foil and maintained in air on a rocker platform at 4 cycles/min so that they were alternately exposed to the culture medium and atmosphere. One-half the volume of the culture medium, including hormone supplements or protein synthesis inhibitors, was changed every other day. Estradiol and cycloheximide were prepared as concentrated stocks in ethanol or water, respectively. To measure percent inhibition of protein synthesis by cycloheximide, newly synthesized protein secreted by cultured liver fragments was radiolabelled by incubating with 5 $\mu\text{Ci/ml}$ [^{35}S]methionine (1200 Ci/mmol) for 1.5 hr at 22°C with rocking. The liver fragments were homogenized and homogenate was centrifuged at 15,000 x g for 30 min. Two ml of homogenate was precipitated with 10% TCA (final concentration) and 0.5 mg BSA as carrier. Precipitate was washed 2X with 5% TCA and then dissolved with 0.3 N NaOH. Ten ml of scintillation solvent was added and samples counted. [^3H]uridine incorporation was measured by precipitation with trichloroacetic acid after a 3 hr labeling period with 5-10 μCi .

Liver cytoplasmic RNA was extracted as described above. Northern and dot blot analysis were performed to determine mRNA levels for vitellogenin, albumin, fibrinogen, transferrin, clone 12B, ferritin, and globin after treatment with estradiol or metabolic inhibitor.

In vitro Synthesis of ^{32}P -labelled mRNAs

Plasmids containing cDNAs were linearized by appropriate restriction at polylinker sites in the vectors 3' to the cloned fragments. Transcription reactions were carried out for 1 hr at 37°C in a mixture of 1 μg of linearized DNA template, 40 mM Tris-HCl pH 8.0, 8 mM MgCl_2 , 2 mM spermidine, 50 mM NaCl, 0.4 mM ATP, CTP, GTP and 0.04 mM UTP, 12 mM dithiothreitol, 10 units of

RNasin, 10 μ Ci of [γ - 32 P] UTP and 40 units of T7 or T3 polymerase. The reaction was stopped by the addition of 1 unit of RNase-free DNase for 15 min at 37°C. The mRNA was extracted with phenol and precipitated with ethanol three times. To synthesize 5'-capped mRNAs, transcription reactions also included 0.4 mM m⁷G(5')ppp(5')A, and 0.04 mM GTP.

Cell-Free System for mRNA Degradation

Preparation of Extracts

Post-mitochondrial extracts (excluding nuclei, mitochondria and lysosomes) consisting of cytosol and polysomes were prepared from both control and estrogen-treated frogs. A second extract including only polysomes was also prepared. One or two frogs were typically used in the preparation of each extract. Livers were excised and perfused with sterile 1X SSC and then washed in appropriate homogenization buffer. Post-mitochondrial homogenization buffer consisted of 0.25 M sucrose; 30 mM Tris-HCl, pH 7.4; 2 mM dithiothreitol; 1 mM EDTA; 0.1 mM PMSF, while the polysome buffer (buffer A) consisted of 30 mM Tris-HCl, pH 7.4; 1.5 mM magnesium acetate; 1.0 mM potassium acetate and 2.0 mM dithiothreitol. Trasylol (1 μ l/ml; 18,000 units/ml) was added to the buffers prior to use. Livers were diced into small pieces (10 mm fragments) and 2.5 volumes homogenization buffer added, and homogenized on ice with 10 strokes at a setting of 40. The homogenate was filtered through a polyamide nylon membrane (Nitex 27621, 0.5 μ m, Tetko). For the post-mitochondrial extract (cytosol and polysomes), homogenate was centrifuged for 10 min at 1000 x g (3000 rpm) in polypropylene tubes in a Sorvall HS4 rotor. The supernatant was then centrifuged for 15 min at 15,000 x g (13,000 rpm) in 5 ml polyallomer tubes using a Beckman SW50.1 rotor.

Supernatants were removed with a syringe and portions taken and stored at -70°C. The soluble extract was fractionated on sucrose gradients. Linear 15-40% (14 ml) sucrose gradients in a buffer containing 30 mM Tris-HCl, pH 7.4; 2 mM DTT; were overlaid on a 1.5 ml cushion of 70% sucrose in the same buffer. Up to 1.0 ml of extract was loaded on each gradient and centrifuged at 4°C in a SW 41 rotor for 3.5 hr at 225,000 x g (36,000 rpm). Gradients were fractionated using an LKB model 2112 fraction collector equipped with LKB peristaltic pump model 2132, UV absorbance monitor model 2138 and chart recorder model 2210. Fractions (0.45 ml) were collected in 1.5 ml microfuge tubes on ice. Fractions were treated with 1% SDS, proteinase K (40 units) and extracted with phenol/chloroform (1:1). The RNA was recovered from the aqueous fraction by precipitation with 2.5 volumes of ethanol. The precipitates were washed with 75% ethanol, dried, dissolved in sterile water, and analyzed by dot blot and Northern blot hybridization.

Preparation of mRNP and Polysomal mRNA

Post-mitochondrial extracts were fractionated using 15-40% sucrose gradients and fractions corresponding to 20-80S mRNPs and heavy polysomes were pooled. Ribonucleoprotein particles and polysomes were suspended at a concentration of 50-100 A₂₆₀ units/ml in buffer A and stored in small aliquots at -70°C. Tubes containing heavy polysomes or mRNPs were thawed immediately prior to an experiment and unused material discarded rather than refrozen.

Preparation of 0.4 M NaCl extracted mRNPs/polysomes and 0.4 M salt extracted proteins

A solution of mRNPs or heavy polysomes (50-100 A₂₆₀ U/ml) was made 0.4 M in NaCl by the addition of 1/10th volume of 4 M NaCl and incubated for 30 min at 0°C. The salt-extracted mRNPs or heavy polysomes were purified by

centrifugation at 4°C in a Beckman TL-100 tabletop ultracentrifuge (TL100.3 rotor) for 1 hr at 165,000 x g (55,000 rpm) through a cushion of 25% sucrose in a buffer containing 20 mM Tris-HCl, pH 7.5; 3 mM magnesium acetate and 0.5 M NaCl. The high salt extracted mRNPs or polysomes were suspended in buffer A and stored at -70°C prior to use. The supernatant proteins obtained after centrifugation of mRNPs or polysomes were dialyzed at 4°C against several hundred volumes of buffer containing 20 mM Tris-HCl, pH 7.5; 150 mM KCl; 1 mM dithiothreitol, 1 mM magnesium acetate and 10% (v/v) glycerol and stored at -70°C.

Preparation of S100 Extracts

Livers were excised, perfused and homogenized, as described, in total extract buffer. Cell debris was removed by centrifuging at 1,000 x g (3,000 rpm) for 10 min at 4°C in a Sorvall HS4 rotor. Supernatant was removed and centrifuged for 15 min at 15,000 x g (13,000 rpm) in a Beckman SW 50.1 rotor. The supernatant was centrifuged for 1 hr at 100,000 x g (45,000 rpm) in a Beckman TL-100 tabletop ultracentrifuge (TL 100.3 rotor) at 4°C. The cytosolic supernatant was stored in portions at -70°C. The pellet (polysomes) was dissolved in buffer A.

In vitro assay conditions

Standard 50 µl reactions were prepared on ice and contained 30 mM Tris-HCl, pH 7.4; 2 mM magnesium acetate; 1 mM ATP and RNA substrate. Nuclease activity (polysomes from sucrose gradients, 0.4M NaCl extracted polysomes) was added last. The RNA substrate was either total RNA (5-20 µg), [³²P]transcripts (10-100 pg albumin or ferritin) or RNA complexed as mRNPs or polyribosomes. After incubation at 22°C for various times, samples were

treated with 1% SDS, proteinase K (150 μ g), extracted with phenol/chloroform (1:1) and the levels of mRNA were quantified by Northern blot analysis probing with the appropriate radiolabeled cDNA as described above, or direct autoradiography of transcript reactions. The recovery of RNA, in all reactions, was determined by adding an anti-sense albumin transcript after the incubation and performing the extractions as described above. Recoveries ranged from 60-100% but were usually greater than 80%. The data were quantified by scanning laser densitometry and were normalized to the signal for the anti-sense albumin transcript.

RESULTS AND DISCUSSION

Chapter I: Estrogen Does Not Induce Covalent Changes in Albumin mRNA

Messenger RNAs can arbitrarily be divided into four segments. These include a 5' noncoding segment, a coding segment, a 3' noncoding segment, and a 3' poly(A) tail. Estrogen could alter the stability of albumin mRNA by acting at any of the many steps in the pathway of RNA metabolism. Any or all of the four segments of albumin mRNA could be affected. Possible mechanisms for the destabilization of albumin mRNA by estrogen could include: 1) the selection of a different transcription initiation site resulting in the loss or introduction of stability determinants at the 5' terminus of the message, 2) alternate splicing of the message such that sequences affecting stabilization might be differentially expressed in the mature transcript, 3) alteration of the transport of the message into the cytoplasm, 4) changes in polyadenylation of either the nuclear precursor or mature message. There is a precedent for all of these possible mechanisms as discussed in the Introduction. This chapter describes the cDNA sequence of both the 68 and 74 KDa Xenopus albumin mRNAs and their derived amino acid sequence. It is determined whether estrogen alters the stability of albumin message through any of the aforementioned mechanisms.

RESULTS

The original albumin cDNA insert from pX1A14 (Schoenberg, 1981) was subcloned in opposite orientations into the Eco RI site of pGEM3Z. These clones were used for the preparation of overlapping deletion fragments by digestion with

exonuclease III and mung bean nuclease (Henikoff, 1984). Double-stranded DNA sequencing of the deletion fragments was performed by a modification of the method of Chen & Seeburg (1985) using oligonucleotide primers complementary to the SP6/T7 promoters of the cloning vector, modified T7 DNA polymerase (Sequenase™) and [³⁵S]-dATP. To bridge gaps in the sequence the same procedure was used with oligonucleotides derived from the preceding sequence. From this analysis, it became clear that the 3' end of albumin cDNA was missing from pX1A14.

Additional clones corresponding to the 68 KDa and 74 KDa albumin were isolated from a λZAP cDNA library constructed by Dr. R. Pastori of this laboratory. The library was screened using a 3'-2.3 kb Eco RI genomic clone isolated by Dr. L. Smith in the laboratory containing a portion of exon 13, exons 14 and 15 and the 3' flanking region as well as oligo(dT) as probes. The sequence of the genomic clone is shown in Figure 1. The clones were sequenced and found to contain the extreme 3' end including the poly(A) tail. A discussion of both sequences follows below.

Restriction Enzyme Analysis

Albumin clones were further characterized by restriction enzyme analysis. The restriction enzyme maps of the two albumins are identical for most restriction enzymes. Different cleavage patterns were observed using Eco RI, Hae III, and Afl III restriction enzymes. The difference in restriction enzyme patterns between the 68 and 74 KDa albumin inserts for Eco RI was useful for quickly screening putative 68 and 74 KDa inserts from the library. The 68 KDa insert digested with Eco RI gave two fragments of approximately 1750 and 250 bp whereas the 74 KDa insert gave three bands of about 1000, 750, and 250 bp.

Figure 1. Sequence of the 3' Terminus and Flanking Region of the Xenopus laevis 68 KDa Albumin Gene.

Xenopus contain at least two albumin genes, encoding peptides of 68 and 74 KDa in size (May et al., 1983). Part of the 68 KDa albumin gene was cloned and by sequence analysis this clone was found to contain a portion of exon 13, exons 14 and 15, and the 3' flanking region as shown. The intron-exon borders were obtained by comparison to the complete sequence of the 68 KDa albumin cDNA (Moskaitis et al., 1989). The sequence presented is that of the terminal 2.3 kb Eco RI fragment of a clone isolated from a Charon 4 library of Xenopus DNA subcloned into pGEM3Z and processed by double-stranded dideoxy sequencing using synthetic oligonucleotides as primers. Exons are underlined.

* This clone was isolated by Dr. Larry Smith in our laboratory.

1

L	K	M	V	Q	K	C	C	T	A	D	E	H	Q	P	C	F	D	T	E
CTTAAGATGG	TACAGAAATG	CTGTACTGCA	GATGAACACC	AGCCATGTTT	TGATACAGAG														
GTATGGCAAC	ACTCTTGTGT	TTTTTGTATC	ATGAAAACAA	CACAGGCTGA	ATGTGTTCAA														
TGTTCAATTG	ATATCCTTTT	CCTTCAACCA	CATTTTACT	TATTTATGTC	TAATCTCATG														
		K	P	V	L	I	E	H	C	Q	K	L	H	P					
TTTTATCTTA	TTTATCCACA	GAAACCAGTA	CTGATTGAAC	ACTGTCAAAA	ACTCCATCCA														
*																			
TAAGAGTCCA	TAAGAGCAAA	GACCAGGTAA	GATTAGATGC	CTAAATCTGC	AACTTTATGC														
AAGTGAATAT	GCATATAGTA	TTCTACAGGG	GGCAAATTAT	AAGGAACAAG	CAATTGAAAA														
TGTGTCTGTA	AATTGCATGA	AAGTGGATAT	AAGGCAAAAA	AAATCAAATC	CCATTCTTTA														
TTTAATGAAA	AAGAAGTCTA	TCTCCAGTAT	TCTTTAATTA	AGAAATTTTT	AAAAATCCCC														
CCTTTGTTTT	ACTTGCTCCG	ACTTCTGTGG	ATAGGAAACT	TCAGATGGTC	CCTAACTAGG														
GATGGGCGAA	TTTGACCTGT	TTCATTTTAC	TATAAATTCG	CCGCTGGCAA	ATGTCGCAGA														
CGGCTATTAA	AGTCTATGTG	CTTAAAAAAA	AAAAAACTT	TTGCGGCACG	AAAATTTTTT														
TGATGTGCGT	CTCTTTTTTT	TTTTTTGACA	CACGCCGCCA	TACAAGTCTA	TGGGCATCAT														
TTTTTCGGCG	AAACTAGGCG	AAAAAATTCA	CCCATCCCTA	TCCCTAACTG	CTCTGCAGGA														
AAACGTATCA	TACTGTTAAA	CTGCAGGGGA	GCTCCTCCAC	CTTACTTCAC	AGACTCGAAC														
AGCTTGTTGT	TCTGTAGAGC	AGCCAGCGAC	CATCTGAGAT	TCGTATCAGT	AGCGATCCTA														
GAGGGGGGCG	GTGCGTGACG	CACAGCCGGG	CCCTGCCCTT	CGTACTGCCA	CATTTACAGC														
GCGCACGGAC	TGCCGGGGGG	CCCTGAGGAC	TGCTCCCCCT	GTAGTTCCGC	CACTGATTCTG														
TATCGACAGA	CCCAGTGCAT	ATCTGATTAT	TAATTAGTCT	TGCTGCATCT	TGCTCTATTG														
TATATTAATA	ATTATATAGA	TATTAATTGT	ACTTTTAAAA	ACTGCTGAGG	CGTTTTGCAT														
TGTGCCAACA	TTTTTATTTA	TTGATATTTA	GTTCTGATTA	ACTACATGTT	TAGTATTTTC														
TAATCATTAG	AACAACCTTA	CTGTCTAAAC	ACCAGCCCAT	CATACATTTG	TCTTAGTTTA														
ACACATTCTC	TTTTGTTTTG	CAGCCTTCAA	ACTCACTGAG	GAACACCTTC	CATCTCTCAA														
ACACAAGAAA	AAAAAGTTCC	TTCAGCTGAA	AAGAGCATTT	GCTTAGAGCA	TTCAACTGTG														
TGTTGTAATA	AATAAAGCAT	TTAAAAAAT	ATATAGAGAG	TGAAAATGTA	TTATCTGTTG														
TTTCAGTGTC	ACATACACAC	CAGAACTATA	TGGTTTACAT	ATTTTAGTCC	GATTTCCGTG														
ATCTCTTTCT	ACCTAGAAAA	TTAAACATCA	GTTAGATAAG	AAGGTACATA	AGAACAAAAT														
GAGAAACCGC	AGGCTAAGAG	TGAGGTGTTT	TTATGAATTA	GGACCAGTAC	AGTGTATTTT														
TATCTGTGTG	GTATCTCTAT	TGAGCAATTA	ACTGGATAGG	ATAGGTGTTT	GTGCAGATAC														
TTTATAACAG	CACCCAATAG	ATAGGTTAAT	TCTGAATATT	GCTCTTAACA	TCACACTTCT														
TTGTAGAATC	TCATACTGTA	GTAGATGTTT	ATAAGTCGTA	TGATAGTAGT	TATATTAAAT														
AATTATAATA	CTGTTGATAC	AGTATTAGTC	CCAATCAAAT	CGATTCCATT	TGGACCTGTC														
TTTCAGTGAA	GACAAATGTA	TACAATACAA	ATATGGCAGC	TAGCAGAAAA	TATCAATTAA														
CATACTGTGG	GTCCGATAAT	CATCAACTGA	TTGAAAGCTG	CATCTTTTGC	ATGGAGGATT														
AAAAAAACGG	TTTCACATTA	CATCAGCATT	TATTTATGTA	GCGCTGTCAA	GATACCCAGT														
GCTTATTACA	TATTCCATAG	TACATGTATG	CTATCCTTTA	TCTGGAAACT	GGTTATTCTG														
AAAGCACCGA	ATTATGGGAA	GACCAACTCC	CATAGAGACC	CCTTATAAGT	AATTCTAATT														
TTTAAAAATC	ATTACTTTTT	TTATCTGTCTG	TAATAAAACA	GTCCCTTTAC	TTGATCATAT														
CTAAGCTGCA	TGAATCCATA	TTAGTGCGAA	AACTATCTTA	TTGGTTGAT	TTCATGTTTT														
AACCAAACCT	AAAGCTATGG	AGATCCAAAC	TACAGAAACA	TCCCTTATCT	GGAAAATCCC														
AGGCCCC																			

Sequence Analysis of the 68 and 74 KDa Albumin cDNAs

The sequences of both the 68 and 74 KDa albumin cDNAs, along with their derived peptides, are shown in Figure 2. Both albumin cDNAs were found to be similar in size (2018 bp for 74 KDa vs 2015 bp for 68 KDa) as well as nucleotide composition. The sequence of the 68 KDa cDNA begins at the cap site (verified by primer extension analysis) and extends through poly(A), whereas the sequence of the 74 KDa albumin lacks 27 nucleotides of 5' flanking sequence. Previous work on the 5' flanking and 5' proximal exon regions of the two Xenopus albumin genes shows extensive homology (38/41 identical) in this region (Schorpp et al., 1988; Sweeney et al., 1987). The sequence around the 68 KDa albumin translation initiation site [CAGUCAUG] corresponds to the consensus sequence derived by Kozak (1987) of CCA(G)CCAUG for such sequences in eukaryotic mRNAs with a purine at position -3 followed by 2 pyrimidines. A comparison of the coding sequences of the 74 KDa and 68 KDa albumin demonstrate a 94% sequence identity with variations occurring usually in the third base (wobble position) of codons.

The leader peptide of both albumins consist of a hydrophobic sequence of 24 amino acids ending with the basic amino acids Lys-Arg. An internal Ser-Arg cleavage signal is present in both sequences and yields a prepeptide of 18 amino acids and a propeptide of 6 amino acids. This processing site is ubiquitous in mammalian albumins and is lacking from α -fetoproteins (Strauss et al., 1978). The number and spacing of Cys residues in Xenopus albumins are virtually identical to that seen in mammalian albumins and are distinct from α -fetoproteins (Law and Dugaiczky, 1981). Similarly, there is a high degree of conservation in the location of Pro residues. Like mammalian albumins, the Xenopus albumins are made up of three repeating domains (Figure 3).

Figure 2. Sequence of Xenopus Serum Albumin cDNAs.

The sequences of the 68 KDa (upper) and 74 KDa (lower) albumin cDNAs are shown, along with the derived amino acid sequence. The pre- and propeptides are shown with a dotted line above the sequence. The mutation converting Lys₂₅₆ to Asn is shown by a star. The domains of the protein were determined by repeats in the sequence and by analogy to the domains in rat albumin. The sequence of the 68 KDa albumin cDNA is complete to the cap site; the 74 KDa cDNA lacks the first 27 basepairs. Identical bases are shown with a dot; gaps represent residues missing from one or the other cDNA.

* This figure was taken from the following publication:

Moskaitis, J.E., Sargent, T.D., Smith, L.H., Pastori, R.L. and Schoenberg, D.R. (1989). Mol. Endocrinol. 3: 464-473.

Figure 3. Amino Acid Sequence, Disulfide Bonding Pattern and Triple Domain Structure of the Xenopus 68 KDa Albumin.

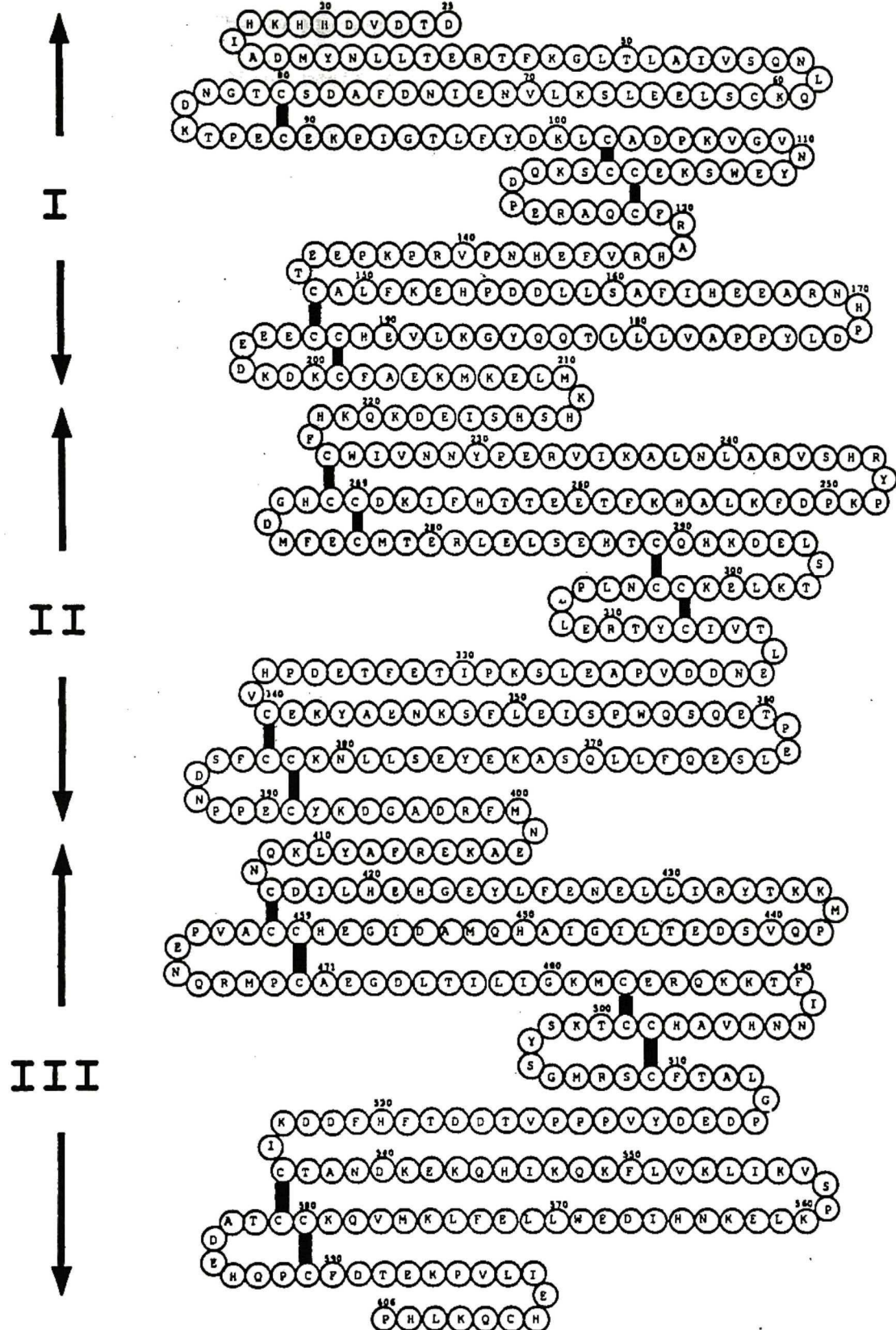
When the amino acid sequence and the disulfide bonding pattern are displayed according to the model of Brown (1976), the spacing of the cysteine residues in the complete polypeptide chains and the positions of the disulfide bridges correspond to that of albumin. The 68 KDa Xenopus albumin sequence can be organized in three similar repeat domains. The amino acids are numbered as in Figure 2. The disulfide bridges are indicated by shaded boxes.

*This figure was taken from the following publication:

Haeffliger, D.N., Moskaitis, J.E., Schoenberg, D.R. and Wahli, W. (1989).

J. Mol. Evol. 29: 344-354.

Reference between 537



The size difference between the two albumin proteins is not evident in their cognate mRNAs. The 74 KDa cDNA alone contains a putative N-glycosylation site Asn-X-Thr at nucleotide positions 805-813 (Figure 2) indicating that the larger protein may indeed be glycosylated and that post-translational processing may account for the different sizes of the two Xenopus albumins. Comparison of the peptide sequences of both Xenopus albumins to those of human, rat and bovine albumins and human, rat and murine α -fetoproteins shows that this site is coincidentally located within three amino acids of a similar N-linked glycosylation site found only in the embryo-specific proteins (Figure 4).

A homology search of each of the protein coding domains of the Xenopus albumins (delineated by the vertical lines in Figure 3) against each other within the same peptide reveals limited sequence conservation with the exception of Cys and Pro. A similar situation is found for rat albumin (Sargent et al., 1981). The peptides encoded by the two albumin mRNAs are virtually identical in domain III, and very similar in domains I and II, with the occurrence of mostly silent mutations in third base positions in the codons. An exception to this occurs in domain II. Here a two base deletion at residue 1081 followed 27 nucleotides downstream by a single deletion causes a shift in reading frame that changes 10 amino acids, two of which are Pro residues.

In Figure 5 the translated sequence of the 68 KDa albumin is compared to the sequence of rat albumin (Sargent et al., 1981). The complete prepropeptides are almost identical in size (606 amino acids for frog, 608 amino acids for rat). Within the predicted open reading frame, both proteins contain a hydrophobic leader sequence of 24 amino acids that share the first 3 amino acids (and their codons), Ser-Arg sequence at the cleavage site between the pre- and propeptides, and a pair of basic amino acids at the cleavage site for the leader peptide. In fact, 11/24 residues (68 KDa albumin) and 8/24 residues (74 KDa albumin) are

Figure 4. Sequence Features of Albumins Related to α -Fetoproteins.

The sequence showing the alteration in albumin protein sequence resulting in the introduction of a consensus site Asn-X-Thr for N-linked glycosylation in the 74 KDa albumin is shown, along with the same regions of mammalian albumins and α -fetoproteins. N-linked glycosylation sites are shown by arrowheads. ABHUS, human serum albumin; ABRTS, rat serum albumin; ABBOS, bovine serum albumin; FPHU, human α -fetoprotein; FPRT, rat α -fetoprotein; FPMS, murine α -fetoprotein. Identical amino acids (single letter code) are shown in capital letters.

* This figure was taken from the following publication:

Moskaitis, J.E., Sargent, T.D., Smith, L.H., Pastori, R.L. and Schoenberg, D.R. (1989). Mol. Endocrinol. 3: 464-473.

GLYCOSYLATION SITES ON 74 KDa ALBUMIN AND α -FETOPROTEINS

	250	260	270	280	290	300
68KDa XALB	SHRYPKPDFK	LAHKFTEETT	HFIKDCCHGD	MFECMTERLE	LSEHTCQHKD	ELSSKLEKCC
		VVV				
74KDa XALB	SHRYPKAEFK	LAHNFTEEV	T HFIKDCCHDD	MFECMTERLE	LTEHTCQHKD	ELSSKLEKCC
ABHUS	250 SqRfPKAEFa	260 evsklvtdlT	270 kvhteCCHgD	280 l1ECaddRad	290 LakyiCenqD	300 siSSKLkeCC
ABRTS	250 SqRfPnAEFa	260 eitklatdVT	270 kinKeCCHgD	280 l1ECaddRaE	290 LakymCenqa	300 tiSSKLqaCC
ABBOS	250 SqkfPKAEFv	260 evtklvtdlT	270 kvhKeCCHgD	280 l1ECaddRad	290 LakyiCbbzb	300 tiSSKLkeCk
FPHU	250 SqkftKvnFt ΔΔΔ	260 eiqklvldVa	270 HvhehCCrgD	280 vldClqdgek	290 imsyiCsqqD	300 tLSnKiteCC
FPRT	250 SqkfPKAnit ΔΔΔ	260 eirkldldVa	270 HiheqCCHgn	280 amEClqdges	290 vmtHmCsqqe	300 iLSSKtaeCC
FPMS	240 SqklteAnFt ΔΔΔ	250 eiqklldVa	260 HiheeCCqgn	270 slEClqdgek	280 vmtyiCsqqn	290 iLSSKiaeCC

Figure 5. Comparison between the Sequences of Xenopus and Rat Serum Albumin.

The amino acid sequences of the 68 KDa Xenopus albumin is shown in single letter code compared to rat albumin. Conserved Cys residues are shown with a vertical line; other conserved amino acids are shown with a diamond. X, Xenopus; R, Rat.

* This figure was taken from the following publication:

Moskaitis, J.E., Sargent, T.D., Smith, L.H., Pastori, R.L. and Schoenberg, D.R. (1989). Mol. Endocrinol. 3: 464-473.

The rat albumin sequence has been previously reported:

Sargent, T.D., Yang, M., and Bonner, J. (1981). Proc. Natl. Acad. Sci. USA 78: 243-246.

identical in the leader sequence of both rat and frog proteins. Overall, there is 38% sequence identity between frog and rat albumin, and the homology increases to 47% if conservation of functional groups (Glu vs Asp, Lys vs Arg, Ser vs Thr, Ile vs Leu) is taken into account. The alteration in reading frame at residue 1081 in domain II increases the number of identical amino acids by 5 between the 74 KDa albumin and rat albumin compared to the 68 KDa albumin. Analysis of the Xenopus and rat albumin sequences by the method of Chou & Fasman (1978) confirms the homologies between the predicted secondary structures of these proteins (except for a region containing multiple Pro residues).

Having the complete nucleotide sequence of both albumin mRNAs from nonestrogenized animals, we examined whether estrogen might introduce instability determinants into albumin through changes in the primary sequence in any region of the message (5'untranslated, coding, 3'untranslated, poly(A) tail). Several albumin cDNA clones isolated from a library prepared from poly(A) RNA of an estrogen-stimulated male Xenopus were sequenced. No differences were found between cDNA sequences from RNA isolated from control or estrogen-treated animals. This sequence included partial 5' untranslated region, the complete coding region and 3' untranslated region. Primer extension experiments performed as part of the sequencing of albumin cDNA clones demonstrated that estrogen has no effect on the site of transcription initiation at the 5' end of the message. Furthermore, previous work demonstrated that estrogen had no effect on albumin RNA capping (M.B. Martin, unpublished results). During the time that DNA sequencing was being performed, Dr. Schoenberg was examining whether the 3' end of albumin mRNA, particularly the poly(A) tail, was important in regulating its stability. Estrogen did not cause changes in polyadenylation of albumin mRNA (Schoenberg et al., 1989). These results are discussed below.

DISCUSSION

To begin to examine the mechanism of albumin mRNA destabilization by estrogen, the first step was to sequence both albumin cDNAs. Albumin cDNAs encoding the 68 and 74 KDa proteins were sequenced. Xenopus albumins are composed of three repeating domains. The peptides are virtually identical in domain III and 95% identical in domains I and II. The number and spacing of Cys and Pro residues in Xenopus albumins are highly conserved compared to mammalian albumins. Such conservation would be required for proper folding of the mature protein, since Pro residues force a turn in the peptide backbone, consequently breaking α -helices. There is a two base deletion at residue 1081 followed 27 nucleotides downstream by a single base deletion that causes a shift in reading frame. This frameshift changes ten amino acids between the two albumins, two being Pro residues. This is expected to have an effect on protein structure. The leader peptide of both albumins contain single base differences that are either silent or result in little net change in hydrophobicity.

The difference in size between the two Xenopus albumins may be due to glycosylation of the larger species (Schoenberg, 1981; Westley and Weber, 1982). Proteins are typically glycosylated on Asn, Ser, Thr and Cys residues with varying amino acid consensus sequences directing glycosylation of a specific residue. One such glycosylation consensus sequence that has been reported is Asn-X-Ser/Thr resulting in the N-linked glycosylation to the Asn residue. This consensus sequence was found in only the 74 KDa albumin. It is possible that other glycosylation sites in the 74 KDa protein are present yet are not identifiable because of the weak consensus for O-linked glycosylation site sequences. Additional post-translational modifications may also contribute to the 6000 dalton difference between the two proteins. Our data, however, clearly

demonstrate that the virtually identical size of the two albumin cDNAs excludes the possibility that the difference in molecular weight between the proteins is a result of a difference in size of their respective mRNAs.

Albumin cDNA clones present in libraries from control and estrogen-treated animals were sequenced to determine whether estrogen might introduce instability determinants through changes in the primary sequence of the message. No differences in nucleotide sequence were found in the 5'-untranslated region, coding sequences nor in the 3'-untranslated regions of any of these clones. This leads to the conclusion that estrogen does not induce a destabilization through modification of the sequence of albumin mRNA.

The 3'-processing and polyadenylation of albumin mRNA was examined by Dr. Schoenberg to test whether estrogen might alter these steps. Oligonucleotide-directed cleavage with RNase H was performed to separate the 3' end of albumin mRNA from most of the mature transcript. Samples of total RNA from control animals and estrogen-treated animals were either untreated or digested with RNase H in the presence of the cleavage oligonucleotide or the cleavage oligonucleotide plus oligo(dT)₁₂₋₁₈. The blot was hybridized to the genomic fragment described in Figure 1. Identical RNase H cleavage fragments were observed using RNA from control and estrogen-treated animals (Schoenberg *et al.*, 1989). This is in agreement with sequence data indicating that estrogen does not induce changes in the 3' end of albumin message. Dr. Schoenberg has also determined that the majority of albumin mRNA has a discrete 17 residue poly(A) tail with lesser amounts of a species with 11 adenosine residues (Schoenberg *et al.*, 1989). Estrogen has no effect on the degree of polyadenylation (Schoenberg *et al.*, 1989). Albumin cDNA clones isolated from libraries prepared from RNA isolated from control and estrogen-treated animals contained 17 or fewer adenosines. Furthermore, the sequence of the 3' end of the 68 KDa albumin gene (Figure 1)

contains no alternate splicing or polyadenylation sites, confirming the results obtained above.

The estrogen-mediated changes in albumin mRNA stability are probably mediated through a mechanism distinct from those reported in other systems. As discussed in the Introduction, steroid hormones have been shown to modulate transcription initiation (Klein-Hitpass, 1990), splicing of the message (Green, 1986; Brawerman, 1987), or polyadenylation (Paek and Axel, 1987; Cochrane and Deeley, 1988).

The change in nuclear albumin RNA levels is probably not the result of inhibition in nuclear-cytoplasmic transport. Here, nuclear RNA levels would be expected to accumulate with time after estradiol administration. In addition, cytoplasmic albumin RNA levels are rapidly changing making it difficult to imagine a nuclear transport mechanism being involved. However, experiments with Xenopus oocytes could be used to examine the problem.

Chapter II: Coordinate Regulation of Serum Protein mRNAs

In the Xenopus hepatocyte estrogen stimulates the production of the egg yolk precursor protein vitellogenin (Wallace and Jared, 1968; Follett et al., 1968; Whitliff and Kenney, 1968). Estrogen does so both by increasing the rate of vitellogenin transcription and by stabilizing vitellogenin mRNA (Brock and Shapiro, 1983; Martin et al., 1986). During vitellogenin production, it was observed that the synthesis of many serum proteins were suppressed, most notably albumin (Follett and Redshaw, 1974; Wangh and Schneider, 1982). Dr. Schoenberg's laboratory has shown that cytoplasmic albumin mRNA levels decreased 90% by 12 hours after a single injection of estradiol (Schoenberg et al., 1989). Estrogen

had little effect on the rate of albumin transcription in isolated liver nuclei (Riegel *et al.*, 1986). It was deemed possible that other serum protein-coding mRNAs might be similarly affected by estrogen-treatment. This chapter addresses the selectivity of the estrogen-regulated post-transcriptional destabilization of albumin mRNA. To examine these possibilities, several serum protein cDNAs were tested for coordinate post-transcriptional regulation of their cognate mRNAs, and their primary and secondary structures were analyzed.

RESULTS

Nuclear and cytoplasmic steady-state messenger RNA levels for albumin, γ -fibrinogen, transferrin, clone 12B, ferritin, and vitellogenin were determined after treatment of whole animals or cultured liver fragments with estradiol. Figure 6 shows dot blots of cytoplasmic RNA isolated from control and estrogen-treated liver fragments 1-4 days after adaptation to culture conditions. Messenger RNA levels were quantified by either laser scanning densitometry or liquid scintillation counting of blots and a summary of the results are shown in Figure 7. Steady-state cytoplasmic mRNA levels for albumin, transferrin and clone 12B mRNAs declined most rapidly with a slower decline seen in γ -fibrinogen. Steady-state levels of ferritin mRNA (which encodes an intracellular protein) are relatively unaffected by estrogen. Vitellogenin mRNA levels increased after estradiol treatment of 2, 3, and 4 days and serve as a positive control. Globin was used to normalize mRNA levels since it is unaffected during the vitellogenic response (Martin *et al.*, 1986). In previous studies steady-state levels of actin mRNA were also unaffected by estrogen (Martin *et al.*, 1986). Therefore, those mRNAs which encode secreted (serum) proteins appear to be coordinately regulated by estrogen. In addition, the mRNA encoding the second protein of inter- α -

Figure 6. Effect of Estradiol on Cytoplasmic mRNA Levels in Cultured Liver Cubes.

Livers from hormonally-naive male Xenopus were placed into culture for 48 hr prior to the addition of hormone at time zero, and estrogen was present in the culture medium throughout the time course. Cytoplasmic RNA was isolated at the indicated times ranging from 1 to 4 days of estrogen-treatment and spotted onto a nylon membrane. Steady-state levels of vitellogenin, albumin, γ -fibrinogen, transferrin, ferritin, clone 12B and globin mRNA were determined. Data were normalized against globin mRNA levels which has previously been demonstrated to be unaffected by estrogen administration (Martin et al., 1986).

Figure 7. Estrogen Effects on Steady-State mRNA Levels in vivo and in Cultured Liver Cubes.

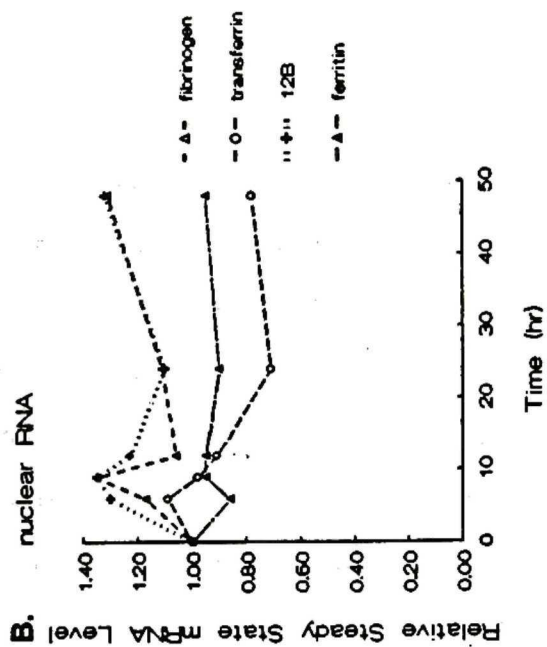
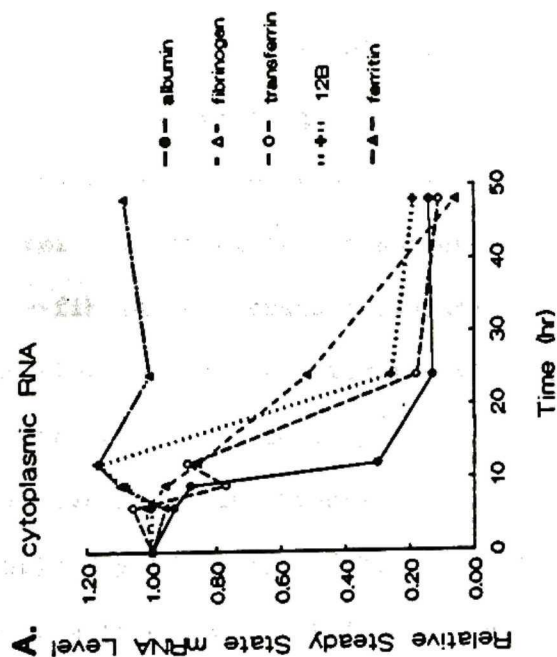
A. & B. Male Xenopus laevis were injected with 1 mg of estradiol at time 0 and groups of 2 animals were sacrificed at the indicated times.

C. & D. Liver cubes were placed into culture for 48 hr prior to the addition of estradiol at time 0 and samples harvested at the indicated times.

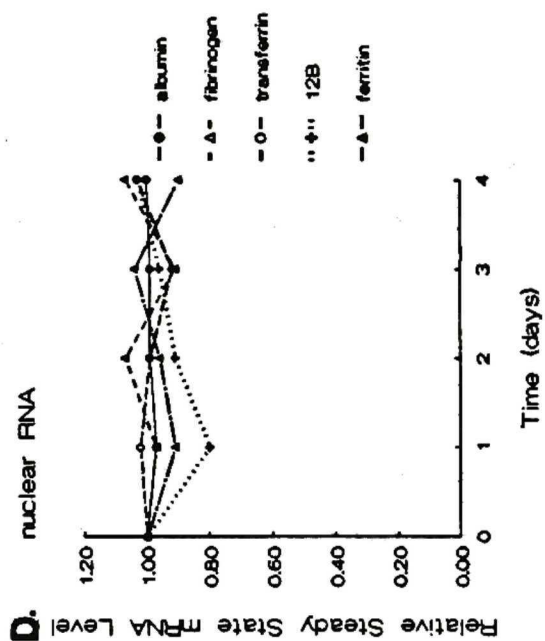
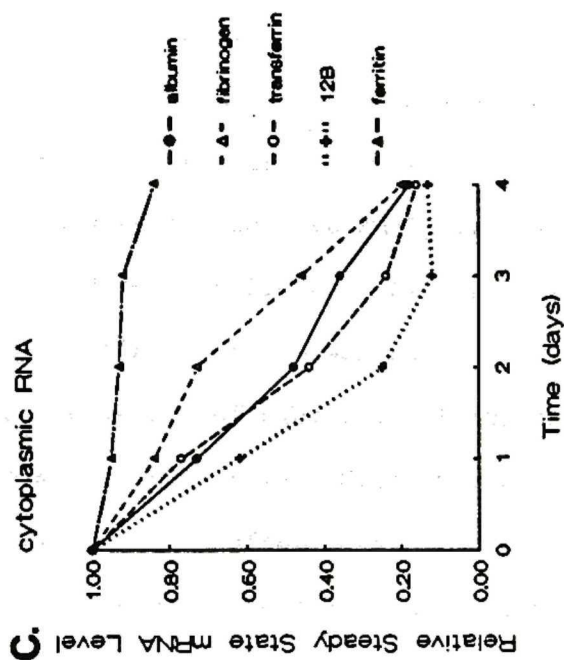
Samples were processed for the isolation of cytoplasmic (A & C) or nuclear (B & D) RNA. Replicate samples were hybridized to the indicated cDNA clones and the relative amount of each mRNA was quantified by laser scanning densitometry of autoradiographs or by scintillation counting of individual dots. The relative amount of each mRNA was normalized to the signal for β -globin, which has previously been demonstrated to be unaffected by estrogen administration (Martin et al., 1986). The data represent the mean of duplicate or triplicate determinations.

-- • -- albumin mRNA; -- Δ -- γ -fibrinogen mRNA; -- O -- transferrin mRNA;
-- + -- clone 12B mRNA; -- \blacktriangle -- ferritin mRNA.

IN VIVO



CULTURED LIVER CUBES



trypsin inhibitor disappears from the cytoplasm after estrogen administration (unpublished data, Schoenberg lab). The overall rates of disappearance of each mRNA in cultured liver fragments are slower than that seen in the liver in vivo. This is not surprising as similar differences between the kinetics of estrogen effects in vivo and in culture have been previously reported (Wolffe et al., 1985; Green and Tata, 1976). Northern blots were also performed on cytoplasmic RNA samples used in the blot analysis. Albumin, globin, fibrinogen, transferrin, ferritin, 12B and vitellogenin RNA were all found to be intact.

A similar approach was used to measure estrogen effects on nuclear steady-state levels of albumin, γ -fibrinogen, transferrin, clone 12B, and ferritin mRNAs (Figure 7). Nuclear steady-state levels of each of these mRNAs were fairly constant after treatment of cultured liver cubes with estradiol for 1, 2, 3, or 4 days. The greatest fluctuations of nuclear RNA levels from control (no estradiol) was 20% for clone 12B. This decrease in nuclear RNA levels was not sufficient to account for the large decrease observed in cytoplasmic steady-state mRNA levels. The results showing little effect on nuclear mRNA in liver explant culture resembled results from livers of Xenopus treated with a single injection of estradiol (Figure 7B). While there are slight fluctuations in the overall nuclear levels of the mRNAs over the time course, the pattern observed reflects relative stability of nuclear mRNA. These data strongly suggest that albumin, γ -fibrinogen, transferrin and 12B mRNAs are all regulated by posttranscriptional destabilization in response to estrogen.

To test if the plasma protein mRNAs that are coordinately regulated by estradiol have a unique primary or secondary structure in common that could target their degradation, γ -fibrinogen (complete), transferrin (complete), ferritin (complete), clone 12B (30%), and the second protein of inter- α -trypsin inhibitor (50%) cDNAs were sequenced. These sequences are shown in Figures 8-12.

Figure 8. Sequence of Xenopus γ -Fibrinogen cDNA.

The sequence of γ -fibrinogen is shown, with the derived peptide sequence below. The location of the two degenerate oligonucleotides used to isolate the clone from the cDNA library are underlined. The cloned cDNA includes most of the 5' untranslated region and the coding sequence and terminates at poly(A). The sequence surrounding the translation initiation site CCATAATG fits the Kozak (1987) consensus sequence for the eukaryotic translation start site of CCA(G)CCAUG with a purine at position -3. By analogy to mammalian γ -fibrinogen, the first hydrophobic stretch of 25 amino acids (MTRLPKQGLLLLQSLALLSSAFGNI) was assigned to the signal peptide sequence. There is a single open reading frame, and the consensus polyadenylation signal AATAAA is found 20 nt upstream from poly(A).

* This figure is taken from the following publication:

Pastori, R.L., Moskaitis, J.E., Smith, L.H. and Schoenberg, D.R. (1990).

Biochemistry 29: 2599-2605.

GGAATTCGGCAGGGACATAGGACCTGCCATAATGACCAGACTTCCCAAGCAAGGATTACTGCTTCTCCAGTCCTTAGCC
MetThrArgLeuProLysGlnGlyLeuLeuLeuLeuGlnSerLeuAla

79

160

CTCCTCTCTAGTGCCTTTGGTAATATTATACCAAACACAGACAACTGCTGCATCTTAGATGGACGTTTTGGGGAGTACTGC
LeuLeuSerSerAlaPheGlyAsnIleIleProAsnThrAspAsnCysCysIleLeuAspGlyArgPheGlyGluTyrCys

241

CCTACAACATGCGGCATTTCCGATTTCTGAACAGATACCAAGAAAATGTTGACACAGACTTGCAGTACCTGGAAAACCTT
ProThrThrCysGlyIleSerAspPheLeuAsnArgTyrGlnGluAsnValAspThrAspLeuGlnTyrLeuGluAsnLeu

322

TTAACTCAAATCAGCAACTCCACAAGTGGAACTACCATAATAGTAGAACATTTAATAGACTCTGGGAAAAAACCTGCGACA
LeuThrGlnIleSerAsnSerThrSerGlyThrThrIleIleValGluHisLeuIleAspSerGlyLysLysProAlaThr

403

TCTCCACAAACAGCCATAGACCCTATGACTCAGAAATCAAAAACATGTTGGATGAAATTAACAGATATGAAAACTATTAT
SerProGlnThrAlaIleAspProMetThrGlnLysSerLysThrCysTrpMetLysLeuThrAspMetLysAsnTyrTyr

484

CAGTATGAAGAAAATATACTATACCTGCAAGAAGTATATTCTTCAAATCAAAAATAAGATTTTCTGCTTAAACAGAAAAATA
GlnTyrGluGluAsnIleLeuTyrLeuGlnGluValTyrSerSerAsnGlnAsnLysIlePheLeuLeuLysGlnLysIle

565

GCAAATCTGGAATTACAATGCCAGCAGCCTTGCCGAGATACAGTTTCAGATACAGGAGTTTCACAGGAAAAGACTGTCAAGAA
AlaAsnLeuGluLeuGlnCysGlnGlnProCysArgAspThrValGlnIleGlnGluPheThrGlyLysAspCysGlnGlu

646

GTTGCTAATAAGGGAGCAAGGCTTAGCGGACTCTACTACATCAAGCCTCTAAAAGCCAAACAGCAGTTCTGTTTTACTGT
ValAlaAsnLysGlyAlaArgLeuSerGlyLeuTyrTyrIleLysProLeuLysAlaLysGlnGlnPheLeuValTyrCys

727

GAAATTGAACCATCTGGCAGTGCATGGACTGTTATTCAAAGAAGACTTGATGGCAGTGTGAATTTCCATAAGAAGCTGGGTC
GluIleGluProSerGlySerAlaTrpThrValIleGlnArgArgLeuAspGlySerValAsnPheHisLysAsnTrpVal

808

CAGTATAGAGAAGGTTTTGGATATCTGTACCAAAACGACAAGACTGAGTTCTGGCTTGGGAATGAAAAATACATCTACTA
GlnTyrArgGluGlyPheGlyTyrLeuSerProAsnAspLysThrGluPheTrpLeuGlyAsnGluLysIleHisLeuLeu

889

AGCACCCAATCTACCATCCCATATGTTATGAGAATTGAGTTAGAAGACTGGAGTAATCAAAGAGCACAGCAGACTATTCC
SerThrGlnSerThrIleProTyrValMetArgIleGluLeuGluAspTrpSerAsnGlnLysSerThrAlaAspTyrSer

970

ACTTTCAGACTGGGTTTCAGAAAAGGATAATTATCGTTTTACTTACGCATACTTCATTGGTGGTGATGCCGGTGATGCCTTT
ThrPheArgLeuGlySerGluLysAspAsnTyrArgPheThrTyrAlaTyrPheIleGlyGlyAspAlaGlyAspAlaPhe

1051

GATGGGTTTGACTTTGGAGATGATCCAAGTGATAAATTCTACACATCTCACAATGGAATGCAGTTTCAGTACTTTTGATAAA
AspGlyPheAspPheGlyAspAspProSerAspLysPheTyrThrSerHisAsnGlyMetGlnPheSerThrPheAspLys

1132

GACAATGACAAGTTTGACGGAACTGTGCTGAGCAAGATGGCTCTGGGTGGTGGATGAACCGATGCCATGCAGCCCCACCTC
AspAsnAspLysPheAspGlyAsnCysAlaGluGlnAspGlySerGlyTrpTrpMetAsnArgCysHisAlaAlaHisLeu

1213

AATGGCAAATACTATCAAGGAGGTACATACAGTGAGGCGGACAGTGGACCTAGCGGCTATGATAATGGTATTATCTGGGCT
AsnGlyLysTyrTyrGlnGlyGlyThrTyrSerGluAlaAspSerGlyProSerGlyTyrAspAsnGlyIleIleTrpAla

1294

ACCTGGAGACGCCGGTGGTACTCCATGAAGTCAGTTACAATGAAAATCATGCCCTTAACAGATATGGAGCAGAAGGACAG
ThrTrpArgArgArgTrpTyrSerMetLysSerValThrMetLysIleMetProLeuAsnArgTyrGlyAlaGluGlyGln

1375

CAGACTTTGGGCGGCTCAAAGAAGTCTGACTTTGAAAACAGAGGAGACTTTTAAGAATCCAGCTTTTGGATTATTATTTA
GlnThrLeuGlyGlySerLysLysSerAspPheGluAsnArgGlyAspPhe

1441

CACTTTATTGTCTTGCTTTATAAATTGTATTACAACTTGTTTTTAAATAAAGATACACCATTTGTG(A)_n

Figure 9. Sequence of Xenopus Transferrin cDNA.

The sequence of transferrin is shown, with the derived peptide sequence beneath it. The cloned cDNA includes most of the 5' untranslated region and the coding sequence and terminates at poly(A). There is a single open reading frame, and the consensus polyadenylation signal AAUAAA is found 14 nucleotides upstream from poly(A).

10	20	30	40	50	60
TTTACCTTCC	AGCCCTGAAT	AATCCAAGGG	CA ATG GAT	TTT TCT CTC CGT	GTG GCA CTG TGC CTG AGC
			Met Asp Phe Ser	Leu Arg Val Ala	Leu Cys Leu Ser
70	80	90	100	110	120
ATG CTG GCC CTG TGC	CTG GCA ATA CAA	AAA GAA AAG CAG	GTG CGC TGG TGT	GTA AAA TCA AAC	
Met Leu Ala Leu Cys	Leu Ala Ile Gln	Lys Lys Lys Gln	Val Arg Trp Cys	Val Lys Ser Asn	
140	150	160	170	180	190
AGT GAA CTC AAA AAG	TGC AAA GAT CTG	GTA GAC ACG TGC	AAA AAC AAG GAA	ATC AAA TTG TCC	
Ser Glu Leu Lys Lys	Cys Lys Asp Leu	Val Asp Thr Cys	Lys Asn Lys Glu	Ile Lys Leu Ser	
200	210	220	230	240	250
TGC GTA GAA AAA TCC	AAT ACT GAT GAG	TGT TCA CTG CTA	TTC AGG AAG ACC	ATG CAG ATG CAA	
Cys Val Glu Lys Ser	Asn Thr Asp Glu	Cys Ser Leu Leu	Phe Arg Lys Thr	Met Gln Met Gln	
260	270	280	290	300	310
TTT GTG TGG ACG GGA	GGG GAT GTG TAC	AAA GGA TCA CTG	CAA CCT TAT AAC	CTG AAG CCA ATC	
Phe Val Trp Thr Gly	Gly Asp Val Tyr	Lys Gly Ser Leu	Gln Pro Tyr Asn	Leu Lys Pro Ile	
330	340	350	360	370	380
ATG GCA GAA AAC TAT	GGT TCA CAT ACA	GAA ACG GAC ACA	TGT TAC TAT GCA	GTG GCA GTT GTA	
Met Ala Glu Asn Tyr	Gly Ser His Thr	Glu Thr Asp Thr	Cys Tyr Tyr Ala	Val Ala Val Val	
390	400	410	420	430	440
AAA AAA TCC AGC AAA	TTT ACA TTT GAT	GAG CTG AAA GAT	AAA AAA TCA TGC	CAT ACC GGA ATT	
Lys Lys Ser Ser Lys	Phe Thr Phe Asp	Glu Leu Lys Asp	Lys Lys Ser Cys	His Thr Gly Ile	
450	460	470	480	490	500
GGC AAG ACT GCT GGA	TGG AAT ATC ATC	ATT GGA TTA CTC	CTG GAG AGA AAG	CTG TTG AAA TGG	
Gly Lys Thr Ala Gly	Trp Asn Ile Ile	Ile Gly Leu Leu	Leu Glu Arg Lys	Leu Leu Lys Trp	
510	520	530	540	550	560
GCA GGG CCA GAT TCA	GAA ACT TGG AGA	AAC GCG GTT TCA	AAA TTC TTC AAA	GCC AGC TGT GTT	
Ala Gly Pro Asp Ser	Glu Thr Trp Arg	Asn Ala Val Ser	Lys Phe Phe Lys	Ala Ser Cys Val	
580	590	600	610	620	630
CCC GGA GCC AAA GAA	CCT AAA TTG TCC	CAG CTG TGT GCC	GGA ATT AAG GAG	CAT AAG TGC TCA	
Pro Gly Ala Lys Glu	Pro Lys Leu Ser	Gln Leu Cys Ala	Gly Ile Lys Glu	His Lys Cys Ser	
640	650	660	670	680	690
CGC TCT AAC AAC GAG	CCG TAC TAC AAC	TAC GCT GGT GCA	TTC AAG TGT TTG	CAA GAT GAC CAG	
Arg Ser Asn Asn Glu	Pro Tyr Tyr Asn	Tyr Ala Gly Ala	Phe Lys Cys Leu	Gln Asp Asp Gln	
700	710	720	730	740	750
GGA GAT GTT GCT TTT	GTG AAG CAA TCA	ACT GTA CCA GAG	GAA TTC CAT AAA	GAT TAT GAG CTG	
Gly Asp Val Ala Phe	Val Lys Gln Ser	Thr Val Pro Glu	Glu Phe His Lys	Asp Tyr Glu Leu	
770	780	790	800	810	820
CTG TGC CCG GAT AAT	ACA AGA AAA AGC	ATA AAG GAA TAT	AAA AAT TGC AAC	TTG GCT AAA GTT	
Leu Cys Pro Asp Asn	Thr Arg Lys Ser	Ile Lys Glu Tyr	Lys Asn Cys Asn	Leu Ala Lys Val	
830	840	850	860	870	880
CCT GCT CAT GCG GTG	TTG ACC AGG GGC	AGA GAT GAT AAA	TCT AAG GAC ATC	ATT GAA TTC CTC	
Pro Ala His Ala Val	Leu Thr Arg Gly	Arg Asp Asp Lys	Ser Lys Asp Ile	Ile Glu Phe Leu	
890	900	910	920	930	940
CAA GAA GCG CAG AAA	ACA CAA GAA TGT	AAA TTG TTC AGG	CTC CCC GGT ATG	GGG AAG GGA TCT	
Gln Glu Ala Gln Lys	Thr Gln Glu Cys	Lys Leu Phe Arg	Leu Pro Gly Met	Gly Lys Gly Ser	
960	970	980	990	1000	1010
AAT TTT CAA GGA CAG	CGC AGT GAG TCT	TAT TCC CCT CCC	ATC TTC TAT GGA	CAG TTT TCT GTT	
Asn Phe Gln Gly Gln	Arg Ser Glu Ser	Tyr Ser Pro Pro	Ile Phe Tyr Gly	Gln Phe Ser Val	
1020	1030	1040	1050	1060	1070
CCT CGG AGC CGA TTA	TTC CAA TGC ATC	CAA GCA CTG AAG	GAA GGG GTG AAG	GAG GAT GAC TCT	
Pro Arg Ser Arg Leu	Phe Gln Cys Ile	Gln Ala Leu Lys	Glu Gly Val Lys	Glu Asp Asp Ser	
1080	1090	1100	1110	1120	1130
GCG GCT CAA GTG AAA	GTT CGT TGG TGT	ACA CAA AGC AAG	GCA GAA AAA ACC	AAG TGT GAT GAC	
Ala Ala Gln Val Lys	Val Arg Trp Cys	Thr Gln Ser Lys	Ala Glu Lys Thr	Lys Cys Asp Asp	

1140 1150 1160 1170 1180 1190 1200
 TGG ACA ACT ATT AGT GGT GGC GCC ATT GAA TGC ACT GAG GCC TCC ACT GCT GAA GAA TGT ATC
 Trp Thr Thr Ile Ser Gly Gly Ala Ile Glu Cys Thr Glu Ala Ser Thr Ala Glu Glu Cys Ile
 1210 1220 1230 1240 1250 1260
 GTA CAG ATT CTG AAA GGT GAT GCT GAC GCA GTC ACT TTG GAT GGA GGC TAC ATG TAC ACT GCA
 Val Gln Ile Leu Lys Gly Asp Ala Asp Ala Val Thr Leu Asp Gly Gly Tyr Met Tyr Thr Ala
 1270 1280 1290 1300 1310 1320
 GGG CTA TGT GGG TTG GTT CCA GTA ATG GGA GAG TAC TAC GAC CAA GAT GAC CTA ACA CCA TGC
 Gly Leu Cys Gly Leu Val Pro Val Met Gly Glu Tyr Tyr Asp Gln Asp Asp Leu Thr Pro Cys
 1330 1340 1350 1360 1370 1380 1390
 CAA CGG AGT TGT TCA CAG GCA AAA GGT GTA TAT TAT GCT GTA GCC ATT GTA AAG AAG GGA ACA
 Gln Arg Ser Cys Ser Gln Ala Lys Gly Val Tyr Tyr Ala Val Ala Ile Val Lys Lys Gly Thr
 1400 1410 1420 1430 1440 1450
 CAA GTC TCA TGG AGC AAT CTT AGA GGA GTA AAG ACA TGT CAC ACT GCT GTT GGA CGT ACA GCT
 Gln Val Ser Trp Ser Asn Leu Arg Gly Val Lys Thr Cys His Thr Ala Val Gly Arg Thr Ala
 1460 1470 1480 1490 1500 1510
 GGC TGG AAC ATA CCC GTC GGA CTA ATT ACC AGT GAA ACC GCG AAC TGT GAT TTT GCT AGT TAC
 Gly Trp Asn Ile Pro Val Gly Leu Ile Thr Ser Glu Thr Ala Asn Cys Asp Phe Ala Ser Tyr
 1520 1530 1540 1550 1560 1570 1580
 GTG GGT GAA AGC TGT GCT CCC GGT TCT GAT GTG AAA TCT AAT CTG TGC GCG CTG TGT ATT GGT
 Val Gly Glu Ser Cys Ala Pro Gly Ser Asp Val Lys Ser Asn Leu Cys Ala Leu Cys Ile Gly
 1590 1600 1610 1620 1630 1640
 GAT CCG GAA AAG CTG TCG GAG AGA GAA AAA AAG TGT TCC CCC AGT GCC AGT GAG GCA TAT TAT
 Asp Pro Glu Lys Leu Ser Glu Arg Glu Lys Lys Cys Ser Pro Ser Ala Ser Glu Ala Tyr Tyr
 1650 1660 1670 1680 1690 1700
 GGA TAC AGC GGT GCA TTC AGA TGT TTG GTC GAG AAA GGG CAA GTG GGT TTT GCA AAA CAC ACC
 Gly Tyr Ser Gly Ala Phe Arg Cys Leu Val Glu Lys Gly Gln Val Gly Phe Ala Lys His Thr
 1710 1720 1730 1740 1750 1760
 ACG GTA TTT GAG AAC ACA GAT GGG AAA AAC CCT GCA GGC TGG GCA AAA GAT CTG AAA TCA GAA
 Thr Val Phe Glu Asn Thr Asp Gly Lys Asn Pro Ala Gly Trp Ala Lys Asp Leu Lys Ser Glu
 1770 1780 1790 1800 1810 1820 1830
 GAT TTT GAG TTG CTG TGC CCC GAT GGC AGC AGA GCG CCC GTC ACT GAT TAT AAG AGG TGC AAC
 Asp Phe Glu Leu Leu Cys Pro Asp Gly Ser Arg Ala Pro Val Thr Asp Tyr Lys Arg Cys Asn
 1840 1850 1860 1870 1880 1890
 CTT GCA GAG GTT CCT GCC CAT GCT GTT GTC ACT TTG CCA GAC AAG AGG GAG CAA GTT GCG AAA
 Leu Ala Glu Val Pro Ala His Ala Val Val Thr Leu Pro Asp Lys Arg Glu Gln Val Ala Lys
 1900 1910 1920 1930 1940 1950
 ATT GTT GTC AAT CAA CAG TCA TTG TAT GGC CGA AAA GGA TTC CAA AAA GAT ATC TTC CAG ATG
 Ile Val Val Asn Gln Gln Ser Leu Tyr Gly Arg Lys Gly Phe Gln Lys Asp Ile Phe Gln Met
 1960 1970 1980 1990 2000 2010 2020
 TTT CAG TCG ACT GGT GGC AAA GAC CTT TTG TTT AAA GAT TCC ACC CAG TGC TTA CTG GAG ATT
 Phe Gln Ser Thr Gly Gly Lys Asp Leu Leu Phe Lys Asp Ser Thr Gln Cys Leu Leu Glu Ile
 2030 2040 2050 2060 2070 2080
 CCA AGC AAA ACC ACA ATG CAA GAA TTC CTT GGA GAT AAA TAT CAC ACT GCT GTT ACC AGC CTG
 Pro Ser Lys Thr Thr Met Gln Glu Phe Leu Gly Asp Lys Tyr His Thr Ala Val Thr Ser Leu
 2090 2100 2110 2120 2130 2140
 AAT AAA TGC AGC ACC TCA AAT GAG GCC TCC TGG CTG CCT GCA CAA TTC CAC AGC TGC ATG AAG
 Asn Lys Cys Ser Thr Ser Asn Glu Ala Ser Trp Leu Pro Ala Gln Phe His Ser Cys Met Lys
 2150 2160 2170 2180 2190 2200 2210
 ATT TAT ATC ATG GTA GAT TGT CCC CTT TCC ATC ATA TAACCCC AAACCTCAGT TTATTCTCAG
 Ile Tyr Ile Met Val Asp Cys Pro Leu Ser Ile Ile
 2220 2230 2240 2250 2260 2270 2280
 ATGTGAAAGG GGGAATTTTT TCCTTATCTT CTGTTGTTTT TGTTTATCCA ATAAAGTTAT ACTCACATGA(A)_n

Figure 10. Sequence of Xenopus Ferritin cDNA.

The sequence of ferritin is shown, with the derived peptide sequence immediately below it. The cloned cDNA includes most of the 5' untranslated region and the coding sequence and terminates at poly(A). Within the single open reading frame the consensus polyadenylation signal AAUAAA is found 16 nucleotides upstream from poly(A). Xenopus ferritin mRNA is larger than that found in Rana or human by approximately 200 nucleotides; the difference being primarily in the length of the 5' untranslated region. A palindromic sequence (underlined) in the 5' untranslated region bears a high degree of similarity to the iron response element described in human ferritins.

* This figure is taken from the following publication:

Moskaitis, J.E., Pastori, R.L. and Schoenberg, D.R. (1990). Nucl. Acids Res. 18: 2184.

1 GGGTCAGAGC TGTCCTCCAA TCCCCACCCT GTCGGTCCCT AGTTCTATGG GTCCCTTGGG
 CTGCAGTACT ACCGGGCCCTT GTTCCCTATT AAATGGTATT TCGTTGATGG TCTAAGCCCG
 ACAGACAGTG CTTGTTGCAC ACACTCATAC ATTCTTGTTT TTGAGAGTTC TTGCTTCAAC
AGTGTGTTGAA CGGAACCTCT CTGAGTCTTT TTTTAGACCA AACTCTCTCT CTCGCATTAC
 240 TCTCTTGCTT TTTGTGACTC TTTTGCGCCA CCAGCAAACA CCGAAGCGCC ATCA ATG
 CAA TCC CAG GTG CTC CAG AAC TTC AAC AGC GAC TGC GAG ATC GCC ATC
 Q S Q V L Q N F N S D C E I A I
 AAC CGG ATG GTG AAC CTG GAG ATG TAT GCC TCC TAT GTC TAC CTG TCC
 N R M V N L E M Y A S Y V Y L S
 ATG TCT TAC TAC TTC GAT CGT GAT GAC GTG GCA CTC CAT CAT GTG GCC
 M S Y Y F D R D D V A L H H V A
 AAG TTC TTC AAG GAG CAG AGT CAC GAG GAA AGG GAG CAC GCC GAA CAA
 K F F K E Q S H E E R E H A E Q
 489 AAC AAA CGT GGG GGC CGT GTC GTC CTT CAG GAT ATC AAG AAA CCA GAG
 N K R G G R V V L Q D I K K P E
 AAG TTC CTC AAA TAC CGT GAC GAA TGG AGT AAC ACC CTG GAA GCC ATG
 K F L K Y R D E W S N T L E A M
 CAG GCC GCT CTG CAA TTG GAG AAG ACC GTG AAC CAG GCC TTG CTG GAT
 Q A A L Q L E K T V N Q A L L D
 CTG CAC AAG CTG GCA TCC GAC AAG GTT GAT CCT CAG CTC TGT GAC TTC
 L H K L A S D K V D P Q L C D F
 CTT GAA TCT GAG TAC TTG GAG GAA CAG GTG AAG GCC ATG AAG GAG CTT
 L E S E Y L E E Q V K A M K E L
 GGA GAC TAC ATC ACC AAC CTG AAG CGC CTT GGG GCG CAG AAT GGC ATG
 G D Y I T N L K R L G A Q N G M
 772 GGC GAG TAC CTG TTC GAC AAG CAC ACC CTG GGG GAG AGT AGC TAAGCGCGCTC
 G E Y L F D K H T L G E S S
 TCAGGGTAGA AACCAACCAGT CAGCTCTCTG TTTCAAAATA CTGTCTCTTA ATATCTGTGT
 GGTATATAACC ATAGCTCCCA TGCCCATGTT CAGACCTTTT CATTTTGAGG ACGATGGGAA
 TATATCTGTG CTCTTAATCA TGCAGAAGCT GCTGTGCTTG TCAACATGTT TTCAATAAAG
 TTTTTCAGC ATTCCAAAAA AAAAAA

Figure 11. Partial Sequence of Xenopus Clone 12B cDNA.

The partial sequence of clone 12B is shown in a 5' to 3' direction. The gap between sequences represents an internal region of clone 12B that has not been sequenced.

This sequence was compared to those in GenBank and no extensive homologies were found and hence it is uncertain what this sequence encodes.

1

CGGGATCGATAAAACAAAGTACGTCATTAAATGTTTAAAAAAGCTAGGTAAAGGGGTTTTTGTGTCTCCATTATGTT
GTAGTTCCTCCTGGTTTAAAAGTTGAACCTCACACCGCCCTCTTTCTTTCATTACCTTGGCCAGTGTAAGCCTCCAA
CCGGCTTCATTTCTTTGCTCACAGTGCGGGCCTGGAAATCA

GGAATTCGGTTGAAGCACAGGGAGGAAGCATACGTGGTCCTAGAATATCCGAATCTAAAGCTGAAGCTGACTGCAAAC
AGGAAAAAACTGGCCAATCTGTTGAGATGAAGACTGGGGTCCCAAATGTCCTTCTGGGTTGCAGAATTCAAGGATTA
ACAGATAAAATGCTGATAAATGCAGTTTGGCAAGAGAATTGAAACAATTAAACAAAAATTAAAGGATTGGAGAATTCA
GTCACAAAGTATAGAT

Figure 12. Partial Sequence of Xenopus Second Protein of Inter- α -Trypsin Inhibitor cDNA.

The partial sequence of the presumed second protein of inter- α -trypsin inhibitor is shown in a 5' to 3' direction. The gaps between sequences represents internal regions of the second protein of inter- α -trypsin inhibitor cDNA that have not been sequenced.

A GenBank search was performed and this sequence was 75% homologous at the nucleotide level to human second protein of inter- α -trypsin inhibitor.

1
AAGCCAGTTATCTGTCAACTTCCTATCAGACCCATTACAAGGAAAACCGTCGACTTCATCAACGATTTAAGGAGAGTC
GATTCTCGACCAACTACCACTCAACTGGGGTTATAACAGACCGTTTCAGTCCCTTATGTACGAACGAGAGGAGTTTAG
TCAAGT

GCTGGAAGTTAGGTGTTTATGAGCATGCTCTCTATATTACCTGGGAGATTAGCAAAGCATTAGAGGTTGATATTTA
TATTACAGAACCACAAGGATTCGGTTTGTTAATGTTCTAAAACAATTACAGAAGATTTGCTGGACTAGTCAAGATTA
CAAAAGGAGATAAAAAGGCTCACATTTCAATTCAAGCCAACCTTTGGATGAGCAGCGAAGTATGTCCACATGCTCCACC
ACAGCTGTGAATGGAACCTTCTAGTAAAGTATGATGTTAAAAGGGAACAAAGTAGCCACCTTCAGGTTTTCAATGGA
TATTTCTCCACTTCTTTGCTCCAGACAGTCTGCCTCCACTTCCTAAGAATATCTTATTTGCATTGATGTTGAGTGGT
CTCCATGTGGGGGCTGAAGATGAAACAGACTGTTCAAGGCTATGAATCTATTTAAGACCCTGCTCGCCCGATGATCAGT
CAGTATTGTTGATTTCAACCACAATATTCGGTGTGGAAGGATGAGTTGGTTTATGCCTCATCTGTAGAAAAAGAAG
ATGCTTCAAAATATGTTCAAAGAATTACGGCCCAATGGAGGTACTAACATTAATGCAAGCCGCTCGAGCAATATTCAA
TCTCA

TTTGGAGTGAGAGTAGTAGCAGCGACTCATGTGAAGAGAACAAAGAGAGTCGTTCTGACCCGAGTTTGAGAAAGTCAT
CAAGTGGCTCCAGCCGACCTAACTCCTCGGAGGAGATAATAATATAGTTTCGAAGAATAAAGTCTTCTTTCTTTATTA
TTTTCTCTGTGTGTTTACCGAAGTACAAAAGAGGGACTTCGGTC

Computer analyses comparing primary sequences of albumin (Figure 2) and the other serum proteins does not reveal any gross common features among these mRNAs that might target this group for degradation. However, these results do not exclude the possibility that a unique sequence or structure at the extreme 5' end (untranslated) could be present and target these mRNAs for degradation. A limitation of the method of cDNA library construction is that 15-60 nucleotides on 5' end of most clones are missing.

A similarity in primary sequence was found between the 68- and 74 KDa albumin mRNAs and γ -fibrinogen mRNA which might allow recognition of regulated mRNAs. Figure 13 shows that the 3' untranslated regions of these three mRNAs are AU-rich, ranging from 65% A + U in the 68 KDa albumin mRNA to 74% in γ -fibrinogen and there is a conserved sequence surrounding the poly(A) addition signal. There is no clear symmetry to this sequence yet the regions of similarity extend both 5' and 3' from the canonical AAUAAA signal. Comparison to two other Xenopus liver mRNAs, vitellogenin (which is synthesized in the hepatocyte) and β -globin (synthesized by other hepatic cells) did not show similar sequence features. This analysis was extended to the other serum protein-coding mRNAs which were also estrogen-regulated but this sequence was not present. Hence, a consensus sequence present on those mRNAs destabilized by treatment with estradiol was not found.

DISCUSSION

It has been reported that estrogen administration to either intact Xenopus or hepatocytes in primary culture resulted in a decrease in synthesis of many serum proteins found in the absence of hormone (Follet and Redshaw, 1974; Wangh, 1982). Work from this laboratory has shown that estrogen regulates

Figure 13. Sequence of the 3' Untranslated Regions of Serum Albumin and γ -Fibrinogen mRNAs.

The sequences of the 3' untranslated region of the 68- and 74 KDa albumin and γ -fibrinogen mRNAs are shown beginning with the termination codon. The relative base composition of each message is tabulated in the second panel. The bottom panel shows the alignment of the sequences surrounding the consensus poly(A) addition signal for these mRNAs compared to β -globin and vitellogenin. Conserved nucleotides in the albumin and γ -fibrinogen mRNAs are shown. There is no apparent symmetry to this sequence, yet it is clear that the regions of similarity extend both 5' and 3' from the canonical AAUAAA signal. Comparison to three other Xenopus liver mRNAs, vitellogenin and transferrin (which is synthesized in the hepatocyte) and β -globin (synthesized by other hepatic cells) failed to demonstrate similar sequence features.

* This figure is taken from the following publication:

Pastori, R.L., Moskaitis, J.E., Smith, L.H. and Schoenberg, D.R. (1990).

Biochemistry 29: 2599-2605.

3' UNTRANSLATED REGIONS

68 KDa albumin UAAGAGUCCAUAAAGAGCAAAAGACCAGUCUUCACAAUCACUGAGGAAACACCUUCCAUUCUCAAACACAAAGAAAAAA
 74 KDa albumin UAAGAGUCCAUUAGAGCAAAAGGCCAGUCUUCACAAUCACUGAGGAAACAUUCUCCAUUCUCCAUU GAAAAAA
 fibrinogen UAAGAAUCCAGCUUUUGGAUUAUUAUUUAACACUUUAUUGUCUUGCUUUUAUAAAUUUCACAAACUUGUUUUUAAAA
 68 KDa albumin GUUUCUCCGUCUGAAAAAGGCAAUUUGCUUAGAGCAUUAACUGUGUGUUAUAAUAAAGCAUUUAAAAAU (A) n
 74 KDa albumin GUUUCUCCAUUCUGAAAAAGAAAUUUG UU CAUUCACUGUCUGUUAUAAUAAAGCGUUUAAAAAU (A) n
 fibrinogen UAAAGAUACACCAUUGUG (A) n

BASE COMPOSITION - 3' UTR

	A	C	G	U	A+U	G+C
68 KDa albumin	41	20	15	24	65	35
74 KDa albumin	37	20	14	29	66	34
fibrinogen	29	14	12	45	74	26

POLY(A) ADDITION SITES

68 KDa albumin CAACUGUGUG UUGUAAUAA - AUAAGCAU U
 74 KDa albumin CAACUGUGUG UUGUAAUAA - AUAAGCAU U
 fibrinogen CAACU-UGUU UUUAAUAA GAUACACCAU U
 consensus CAACU-UGU- UU--AAUAA AUA-A-CAU U
 transferrin GUUUU-UGUUUUAUCCAAUAA GUUACUACUC A
 ferritin UCAACAUGU UUUCAAUAA GUUUUUUCAG C
 β-globin CGUAUCUGCU -CCUAAUAA AAGAAGUUU C
 vitellogenin A2 UGUAAUACAU UUCGAAUAA ACUGUGCAU C

albumin gene expression through changes in the cytoplasmic stability of albumin mRNA (Schoenberg et al., 1989) by a post-transcriptional mechanism (Riegel et al., 1986). Since albumin is only one of many serum proteins whose synthesis is suppressed during the active synthesis of vitellogenin, it seemed possible that a common post-transcriptional mechanism caused the regulation of serum protein gene expression.

A battery of serum protein cDNAs were tested for coordinate post-transcriptional regulation of their cognate mRNAs. Messenger RNA destabilization in response to estrogen was not selective to albumin. Several cloned genes including γ -fibrinogen, transferrin, clone 12B and the second protein of inter- α -trypsin inhibitor were all regulated in the same manner. The data in Figures 6 and 7 indicate that mRNA levels for a number of serum protein-coding mRNAs decrease to low levels in the cytoplasm in response to estrogen. Furthermore, estrogen had little effect on steady-state levels of these RNAs in the nucleus. From this it may be concluded that the most likely mechanism regulating albumin, γ -fibrinogen, transferrin, clone 12B and the second protein of inter- α -trypsin inhibitor gene expression is a post-transcriptional destabilization of their mRNAs in the cytoplasm of the hepatocyte.

The results seen in vivo were reproduced in vitro with cultured liver fragments. The observation of similar results in vivo and in liver explants suggests that estrogen acts directly on the hepatocyte rather than indirectly to produce a generalized coordinate destabilization of serum protein coding mRNAs. The cellular control mechanisms that cause stabilization of vitellogenin mRNA and destabilization of several serum protein-coding mRNAs in vivo must also be functional in the cultured fragments.

To determine whether the decrease in cytoplasmic mRNA levels was due to transcriptional inhibition, our laboratory previously reported rates of

transcription in isolated liver nuclei for the abundant albumin and vitellogenin genes (Martin *et al.*, 1986; Pastori *et al.*, 1990). This same analysis proved unsuccessful for measuring rates of transcription of less abundant genes such as γ -fibrinogen (Pastori *et al.*, 1990). Transcription rates in vertebrate nuclei has been estimated to be 20-30 times lower than in mammalian cell nuclei (Marzluff and Huang, 1984). This limits the use of transcription run-on assays in *Xenopus* liver to only the more actively transcribed genes such as vitellogenin and albumin. However, Dr. Schoenberg has shown that the relative steady-state level of albumin mRNA in the nucleus accurately reflects the transcriptional status of the albumin genes (Schoenberg *et al.*, 1989). Thus measurement of unchanged nuclear mRNA levels of the serum proteins demonstrates that the observed decrease in cytoplasmic mRNA is due to degradation, rather than a decrease in the rate of transcription of these genes.

Shared sequences may be present on the serum protein coding mRNAs that are destabilized after estrogen-treatment. Common sequence elements have been identified in the 5' ends of α_1 -acid glycoprotein and haptoglobulin mRNAs involved in the acute phase response (Dente *et al.*, 1985) and in the 3' end of mRNAs for c-*fos* and several inflammatory mediators (Caput *et al.*, 1986; Shaw and Kamen, 1986; Wilson and Treisman, 1988). Destabilization of the latter mRNAs are directed by 3'-AU rich sequences leading to shortening and eventual removal of the poly(A) tail. These sequence elements impart instability to the mRNA and can interfere with translation (Kruys *et al.*, 1989).

The sequences of both the 68- and 74 KDa albumin, γ -fibrinogen, transferrin, clone 12B, ferritin and vitellogenin mRNA were examined for sequences which they may share that could impart similar regulatory consequences. All of these mRNAs are AU-rich in the 3'-untranslated region but no common motifs could be found in all. The 3' untranslated region of γ -fibrinogen and both

albumin mRNAs share a conserved feature surrounding the poly(A) addition site (Figure 13). Comparison to other estrogen-regulated Xenopus liver mRNAs failed to demonstrate similar sequence features. The sequence AUUUG appears once in the 3'-untranslated region of both albumin mRNAs, and there is a single AUUUA in the 68 KDa and GUUUA in the 74 KDa albumin mRNA at the extreme end of the 3'-untranslated region. There is a repeating motif of UCCA or UCAA and two repeats of GAAAA in both albumin mRNAs. The significance of these features are not known.

Common structural elements have been found in the 5' end of prol(I) collagen mRNAs (Chu et al., 1985) and the 3' terminus of histone and transferrin receptor mRNAs (Georgiev and Birnstiel, 1985; Mullner and Kuhn, 1988). Histone mRNA contains a sequence with the potential to form a stem-loop structure possibly making this region resistant to 3' exonucleases (Georgiev and Birnstiel, 1985). The stabilization of transferrin receptor mRNA requires the 3' untranslated region (Mullner and Kuhn, 1988). This region has the potential to form a series of stem and loop structures in the mRNA. Computer analysis of 5' and 3' untranslated regions of estrogen-regulated serum protein-coding mRNAs failed to identify any outstanding structural features such as stem-loops, palindromes or extended repeats. Estrogen does not induce modification in albumin mRNA primary sequence and hence secondary structure.

Selectivity of certain mRNAs for rapid decay can be explained by the action of specific factors recognizing unique sites on mRNA chains. The recognition site for the degradative factor(s) on the serum protein-coding mRNAs is probably rather subtle, since albumin, γ -fibrinogen and transferrin mRNAs (representing ~75% of plasma proteins) are all being degraded and lack obvious common sequence or structural elements. Previous reports have shown that some ribonucleases have recognition sites of two nucleotides (Beutler et al., 1989).

Similar randomly cleaving endoribonucleases could possibly be involved in the estrogen-regulated mRNA degradation described above. Development of an *in vitro* assay system as described in Chapter V may allow identification of the recognition sites involved in the decay of these mRNAs.

One common feature of the mRNAs destabilized by estrogen (albumin, fibrinogen, transferrin) was a short poly(A) tail length. A summary of poly(A) analysis performed by Dr. Schoenberg on regulated and unregulated mRNAs is shown in Table I. Those mRNAs that are destabilized upon estrogen-treatment had poly(A) tails of 16-30 nucleotides in length. Ferritin mRNA which is relatively unaffected by estrogen-treatment has a broader range of poly(A), ranging from 40 to 60 residues similar to that of the stable β -globin (Merkel *et al.*, 1975). Not all liver mRNAs have a short poly(A) tail. Analysis of the A2 vitellogenin mRNA which is induced upon estrogen-treatment yielded a large poly(A) tract of 35-215 residues, with an average of 135. This length is similar to most eukaryotic mRNAs and is in good agreement with published data obtained by solution hybridization (Shapiro and Baker, 1977). At least one intracellular mRNA (pXref-1) has a poly(A) tail of greater than 200 residues and is unaffected by estrogen. Hence, poly(A) tail length may be correlated with estrogen-regulated mRNA stability.

Although the poly(A) tail length for the destabilized mRNAs were not changed upon estrogen-treatment (Pastori *et al.*, submitted), there could be a limited pool of a pre-existing RNA binding protein such as poly(A)-binding protein (PABP) whose presence in a complex with mRNA is necessary for stability. Competition between newly synthesized mRNAs such as vitellogenin and preexisting mRNAs within the cell after estrogen-treatment could result in destabilization of those mRNAs which lose this protein from mRNP complexes. There are several published reports indicating a major role for PABP in the regulation of c-myc and

Table I: Degree of mRNA Polyadenylation

<u>mRNA</u>	<u>Number of Residues</u>	<u>Estrogen Regulated</u>
albumin	17	+, degraded
γ -fibrinogen	30	+, degraded
transferrin	<16	+, degraded
ferritin	40-60, average 45	-, unaffected
vitellogenin	35-215, average 135	+, stabilized
pRef	>200	-, unaffected

Messenger RNAs were hybridized to a DNA oligonucleotide complementary to a sequence 100-400 nucleotides 5' to the end of the message in the presence or absence of oligo(dT). Upon cleavage with RNase H a 3' fragment is generated that either retains or has lost poly(A). The size difference between digests in the presence or absence of oligo(dT) indicates the length of the poly(A) tail. The data from this approach are tabulated.

This analysis was performed by Dr. Schoenberg and has been submitted for publication:

Pastori, R.L.⁺, Moskaitis, J.E.⁺, Buzek, S.W. and Schoenberg, D.R. (1990).

"Coordinate estrogen-regulated instability of serum protein-coding messenger RNAs in Xenopus laevis."

⁺ These authors contributed equally to this work.

c-fos mRNA stability (Brewer and Ross, 1988; Wilson and Treisman, 1988; Bernstein et al., 1989). Since the serum protein-coding mRNAs in this study had short poly(A) tails they are capable of binding only a single molecule of PABP (Sachs et al., 1987) while vitellogenin poly(A) can bind up to 9 molecules of PABP (based on a packing density of 1 per 25 residues). Thus, if PABP was limited, the serum protein-coding mRNAs might be more exposed to ribonuclease activity than mRNAs binding multiple PABPs. Experiments are in progress to determine PABP levels in the hepatocyte and the role, if any, it plays in the estrogen-regulated instability of serum protein-coding mRNAs.

Chapter III: Effect of Metabolic Inhibitors on Albumin mRNA Degradation:

A major question of the post-transcriptional destabilization of albumin mRNA is whether this is a primary response to hormone, or whether estradiol induces a new gene product that later interacts with albumin mRNA to enhance its degradation. This chapter addresses the role of protein synthesis in albumin mRNA degradation. The kinetics of albumin mRNA disappearance was followed in cultured liver cubes that were exposed either to cycloheximide or to MDMP, specific inhibitors of elongation or initiation of protein synthesis.

RESULTS

A. Effect of Protein Synthesis on Albumin mRNA Degradation:

The two inhibitors used to address the role of ribosome binding and translation on the estrogen-dependent destabilization of albumin mRNA were cycloheximide and 2-(4-methyl-2,6-dinitroanilino)-N-methylpropionamide (MDMP).

Cycloheximide freezes the translating ribosome onto the mRNA in the polysome complex. The data in Figure 14 confirm this effect in Xenopus liver explant cultures. Cycloheximide caused a net redistribution of ribosomes into polysome complexes and a similar redistribution of albumin mRNA. MDMP has been shown to block translation initiation by preventing the binding of the 60S ribosomal subunit to the 40S pre-initiation complex (Weeks and Baxter, 1972). The effect of MDMP on the relative distribution of material from the 20-80S peak of mRNP particles, ribosomes and ribosomal subunits, and polysomes is shown in Figure 14. MDMP resulted in a significant loss of material from the polysome fraction of the gradient with redistribution to the 20-80S peak as seen in albumin mRNA levels.

The protein synthesis inhibitors did not appear to be toxic to the cultured liver explants. The concentrations of inhibitor used were those shown by titration experiments to inhibit 90-95% of [³⁵S]methionine incorporation (Table II) yet allow the liver cells to recover completely after removal of the inhibitor. After removal of inhibitor, mRNA levels returned to control and the messages were still found to be intact as seen from Northern blot analysis. The inhibitors affected [³H]uridine incorporation; MDMP inhibited uridine incorporation 25% while cycloheximide inhibited uridine incorporation 8%.

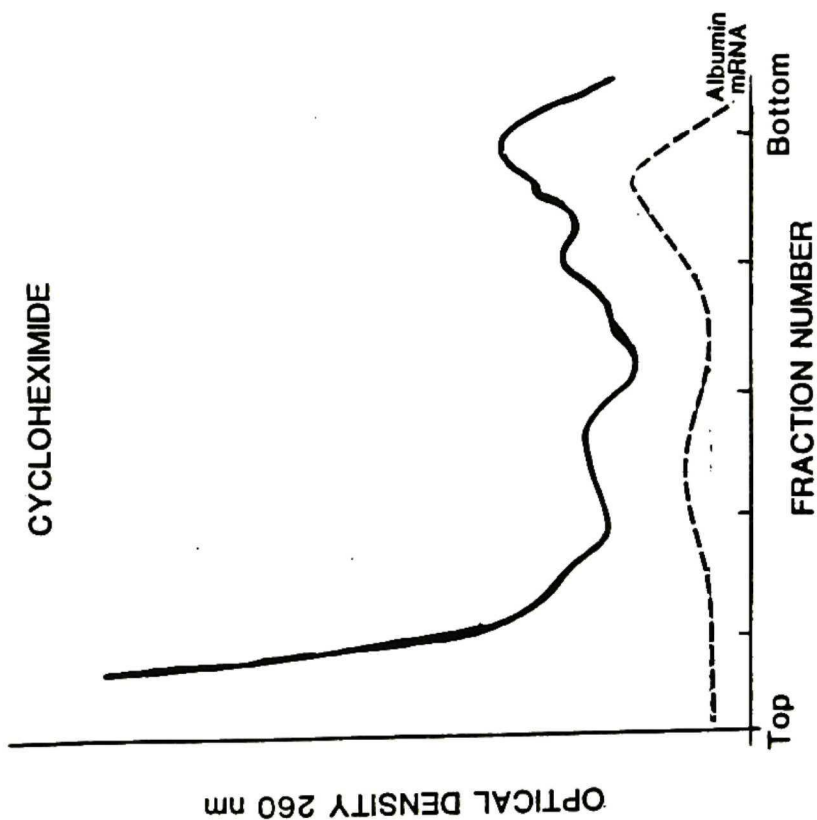
Two experimental protocols were employed. In the first set cycloheximide or MDMP were added to cultured liver cubes at the same time as estradiol to determine whether the destabilization of albumin mRNA required new protein synthesis. Theoretically, the inhibitors should prevent the translation of any newly transcribed mRNA which might encode a protein(s) which targets albumin mRNA for destabilization in the cytoplasm. The second protocol involved addition of cycloheximide or MDMP 30 hr after estradiol, when cytoplasmic albumin mRNA levels had declined about 25%. If polysome binding is required for the destabilization of albumin mRNA then delayed addition of MDMP should return albumin mRNA to

Figure 14. Effect of Cycloheximide and MDMP on Optical Density Profiles and Albumin mRNA Distribution.

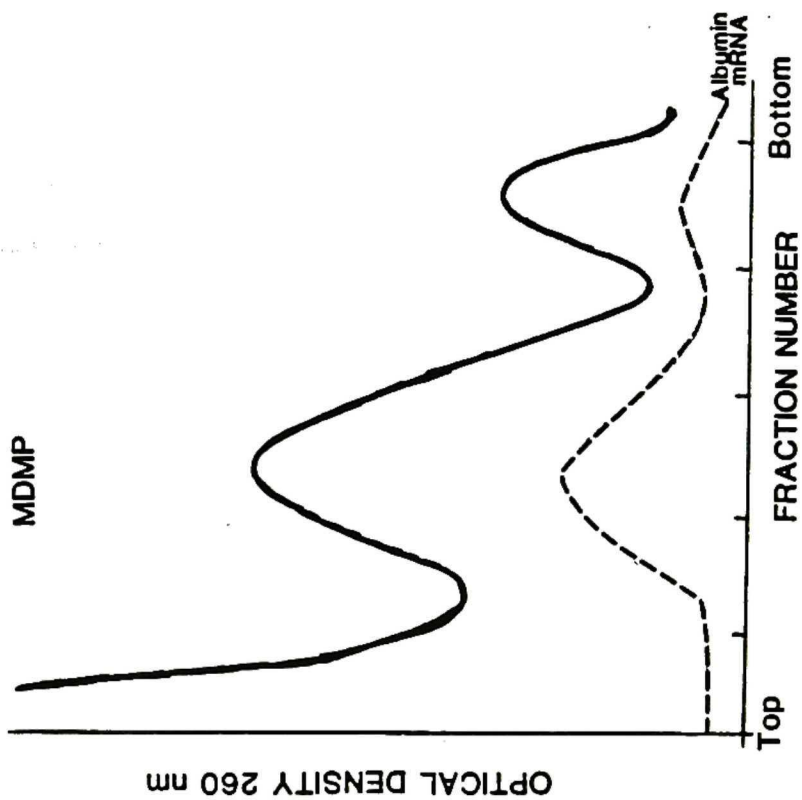
Post-mitochondrial liver extracts were prepared from 72 hr cycloheximide and 72 hr MDMP-treated male Xenopus. Albumin mRNA distribution was examined by sedimentation of extracts through linear 15-40% sucrose density gradients and measurement of optical density of gradient fractions monitored at 260 nm. The relative amount of albumin mRNA was determined by dot blot analysis of RNA extracted from each fraction of the gradient. The data was quantified by scanning laser densitometry.

SEDIMENTATION PROFILES OF LIVER CUBE EXTRACTS

CYCLOHEXIMIDE



MDMP



stabilization
potent

Table II: Inhibition of Protein Synthesis and Uridine Incorporation by Cycloheximide and MDMP in Liver Cube Experiments.

<u>Inhibitor</u>	% Inhibition of Incorporation	
	<u>[³⁵S]methionine</u>	<u>[³H]uridine</u>
Cycloheximide (2 μ g/ml)	92 \pm 6	8 \pm 3
MDMP (20 μ g/ml)	95 \pm 5	25 \pm 8

The concentrations of inhibitor used were those shown by titration experiments to inhibit 90-95% of [³⁵S]methionine incorporation (cycloheximide, MDMP) in liver explant cultures. Values represent the mean \pm standard error from three separate experiments.

control levels. Similarly, if destabilization requires polysome binding, delayed addition of cycloheximide should potentiate the disappearance of albumin mRNA.

The data in Figure 15A show that cycloheximide alone had little or no effect on the steady-state level of albumin mRNA over a 3 day interval. In the same interval, estradiol present in the medium resulted in the disappearance of 75% of albumin mRNA (Figure 15A). The addition of cycloheximide along with estradiol had no effect on the hormone-induced disappearance of albumin mRNA. The addition of cycloheximide 30 hr after estradiol gave a curve for the disappearance of albumin mRNA that was indistinguishable from the curve for estradiol alone.

Unlike cycloheximide, the addition of MDMP to cultured liver cubes caused the loss of some albumin mRNA from the cytoplasm (Figure 15, panel B). This may be due to the partial inhibition of transcription as shown by the 25% decrease in uridine incorporation observed with this inhibitor. However, addition of MDMP with estradiol at the beginning of the experiment had no effect on the cytoplasmic destabilization of albumin mRNA. The delayed addition of MDMP in Figure 15, panel B (30 hr after estradiol) also had no effect on the estrogen-induced destabilization of albumin mRNA. Vitellogenin mRNA was induced by estradiol regardless of the presence of either of the inhibitors (data not shown), a result confirming previously published data indicating the induction of vitellogenin to be a primary response to hormone (Brock and Shapiro, 1983).

From these experiments one can conclude that inhibition of translation by 90-95% had no effect on the destabilization of albumin mRNA. Unlike several other mRNAs (tubulin, histone, c-myc), the destabilization of albumin mRNA is independent of translation.

Figure 15. Effect of Protein Synthesis on Albumin mRNA Degradation.

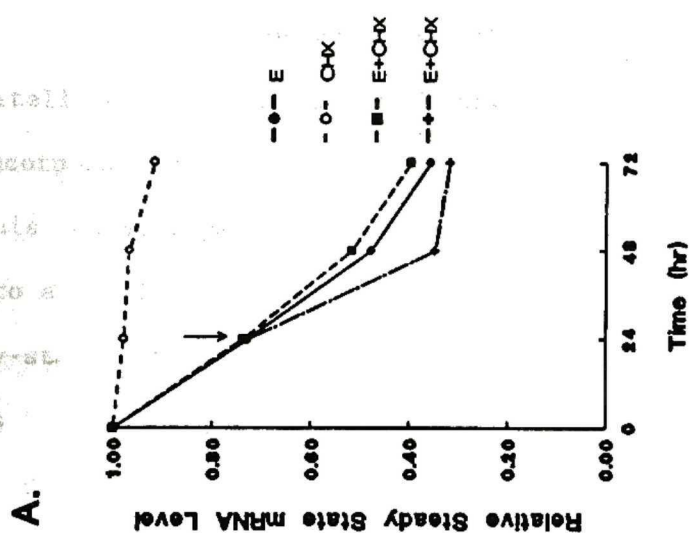
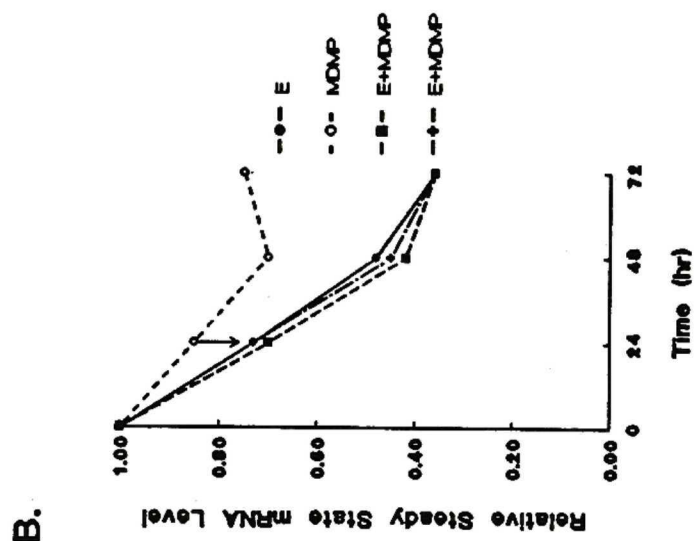
Two protein synthesis inhibitors were used: cycloheximide (panel A) and 2-(4-methyl-2,6-dinitroanilino)-N-methylpropionamide (MDMP, panel B). Two experimental protocols were employed. In the first set, cycloheximide or MDMP were added to cultured liver cubes at the same time as estradiol. The second protocol involved addition of cycloheximide or MDMP 30 hr after estradiol, when cytoplasmic albumin mRNA levels had declined approximately 30%.

Cytoplasmic RNA was isolated at the indicated times ranging from 24 to 72 hr and spotted onto a nylon membrane. Steady-state levels of albumin and globin mRNA were determined. Data was normalized against globin mRNA levels which has previously been demonstrated to be unaffected by estrogen administration (Martin *et al.*, 1986). Data are shown relative to nonestrogenized liver cubes at each of the time points.

-- • -- estrogen-treatment alone; -- O -- cycloheximide treatment alone (A) or MDMP treatment alone (B);

-- ■ -- estrogen + cycloheximide treatment throughout (A) or estrogen + MDMP treatment throughout (B);

-- + -- estrogen + delayed addition cycloheximide (A) or estrogen + delayed addition MDMP (B).



B. Effect of a Limited Pulse of Estradiol on the Loss of Albumin and Accumulation of Vitellogenin mRNA:

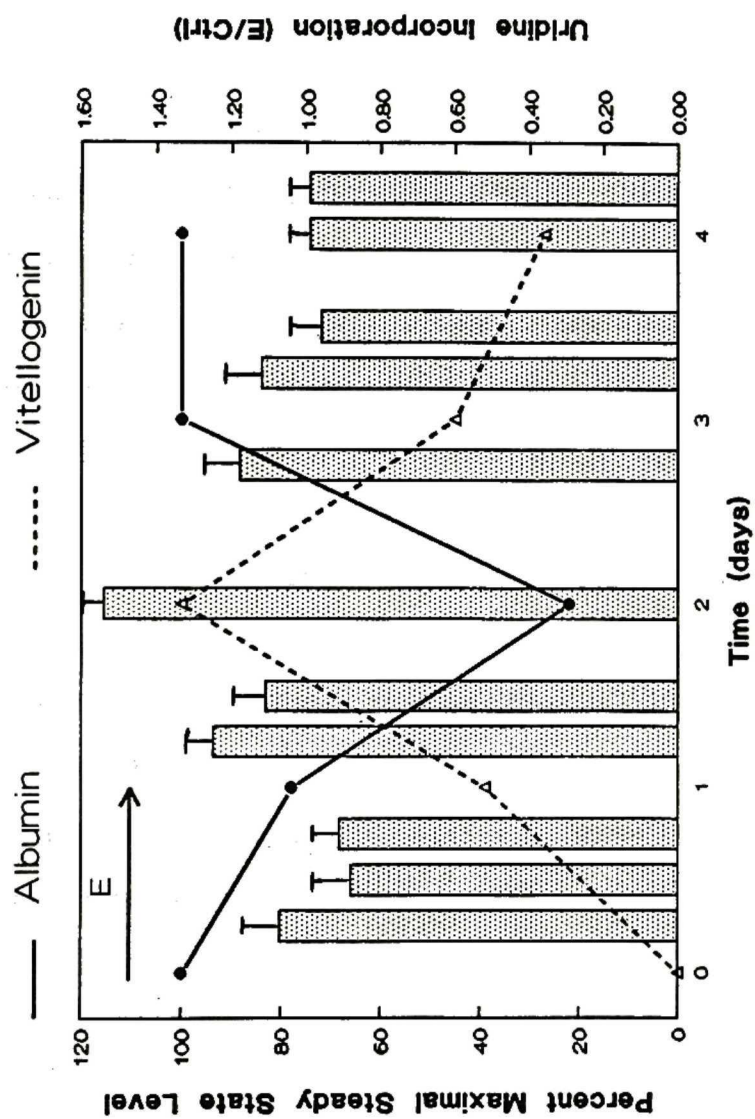
The relationship between the induction of vitellogenin mRNA and the consequent overall changes in total transcriptional activity (Martin et al., 1986) and the destabilization of the serum protein-coding mRNAs was determined. Most experiments published to date which use primary hepatocyte or liver explant cultures have either employed conditions in which estrogen is present throughout the experiment, or conditions of maximal stimulation followed by abrupt withdrawal of hormone from the medium. The effects of a limited "pulse" of estrogen (one that should not maximally induce vitellogenin expression, but should initiate both this process and albumin destabilization) were examined. In the experiment shown in Figure 16, liver cubes from hormonally-naive male Xenopus were exposed to estradiol for 30 hr, after which the medium was replaced and the cultures were maintained without hormone. The relative steady-state levels of cytoplasmic vitellogenin and albumin mRNA were then determined, as was [³H]uridine incorporation.

The pulse of estrogen resulted in the expected decrease in cytoplasmic albumin mRNA to a level 18% of control by 2 days after which it returned to control steady-state levels. A similar plot of vitellogenin mRNA resulted in a curve that was almost the inverted image of that seen for albumin, with a maximal induction at 2 days and return to 20% of maximal by 4 days. In keeping with this pattern, there was a 50% increase in uridine incorporation by 2 days that later returned to control values.

The data in Figure 16 indicates there is some relationship between the disappearance of albumin mRNA and the induction of vitellogenin mRNA, at least temporally.

Figure 16. Effects of a Limited Pulse of Estradiol on the Loss of Albumin and Accumulation of Vitellogenin mRNA.

Liver cubes from male Xenopus were cultured for 48 hr prior to the addition of 10^{-6} M estradiol to the medium. Estradiol was present for precisely 30 hr (arrow) after which the medium was changed to one lacking hormone. Cytoplasmic RNA was isolated from the cultures at the indicated times. Replicate samples were hybridized to the indicated cDNA clones and the relative amount of each mRNA was quantified by laser scanning densitometry of the autoradiographs. The data were normalized to the signal for β -globin and the data represent the mean of triplicate determinations. [3 H]uridine incorporation (bars) was determined by a 3 hr pulse ending at the indicated times. Uridine incorporation is shown as the ratio of that seen at the indicated time after the initiation of estrogen-treatment to that seen in control cultures. Error bars represent standard error from the mean from three separate experiments.



DISCUSSION

The cytoplasmic destabilization of albumin mRNA in response to estrogen requires a lag of at least 12 hr after administration of hormone to male Xenopus (Schoenberg et al., 1989), and is dependent on the action of the estrogen receptor, a well characterized transcriptional activator (Riegel et al., 1987b). From these data our laboratory had originally postulated that the posttranscriptional regulation of albumin mRNA follows a pathway in which estrogen activated the transcription of a quiescent gene whose protein product ultimately targeted albumin mRNA selectively in the cytoplasm for degradation.

To test whether translation was actually required in the destabilization of albumin mRNA, the inhibitors cycloheximide and MDMP were employed. The addition of cycloheximide, an elongation inhibitor, concurrently with estradiol had no effect on the destabilization of albumin mRNA (Figure 16, panel A). These results are in good agreement with the observations of Wolffe et al. (1985), who found that the addition of cycloheximide to primary hepatocytes in culture had little effect on the disappearance of albumin mRNA in response to estradiol. If cycloheximide was added to the culture medium 24 hr before estradiol, the results were the same as those for concomitant addition (data not shown). MDMP, an inhibitor of 60S ribosomal subunit binding to the preinitiation complex, also had no effect on the destabilization of albumin mRNA when added with estradiol (Figure 16, panel B). From these data, one can conclude that the destabilization of albumin mRNA is independent of protein synthesis and hence is a primary response to estradiol.

Vitellogenin stabilization or destabilization in the presence or absence of estrogen was also shown to be independent of protein synthesis in Xenopus (Brock and Shapiro, 1983). Although estrogen action is usually mediated by

initiation of transcription and production of new proteins, the inhibitor studies suggest that synthesis of a new protein is not required for albumin mRNA destabilization. There exists the possibility that a protein with a long turnover is produced upon estrogen-treatment in the delayed addition experiments and causes the degradation of albumin mRNA. However, results from experiments with simultaneous addition of estrogen and inhibitor tend to rule this out.

Interpretation of experiments using protein synthesis inhibitors has often been complicated by secondary toxic effects of these compounds. The failure to observe a biological response following administration of inhibitor may represent either a requirement for protein synthesis in that response or abolition of the response due to overall systemic toxicity. Protein synthesis was inhibited greater than 90% and therefore residual protein synthesis is unlikely to be responsible for the observed responses seen. Albumin and vitellogenin mRNAs were found to be intact for 10 days in liver cells using the cultured liver cube system previously described (Brock and Shapiro, 1983; Wolffe *et al.*, 1981). Puromycin, another elongation inhibitor of protein synthesis, was also used but the inhibitor alone decreased albumin mRNA levels significantly and suppressed the induction of vitellogenin mRNA levels upon estrogen treatment. Hence results from these experiments were difficult to interpret and consequently are not presented here.

Although protein synthesis is not required for the destabilization of albumin mRNA, the estrogen receptor seems to play a role in the process (Riegel *et al.*, 1986; Pastori *et al.*, 1990). Although the estrogen receptor is synthesized in the cytoplasm of the cell, it is unlikely that the estrogen receptor itself interacts with albumin mRNA to affect its destabilization. There are far more albumin mRNA molecules than estrogen receptor molecules in a given liver cell; therefore the receptor would have to act catalytically to effect the

change seen in message stability. Given that virtually all of the Xenopus liver estrogen receptor molecules are nuclear this scenario seems unlikely.

The disappearance of albumin mRNA is related, at least temporally, to the accumulation of vitellogenin mRNA and the estrogen-induced increase in uridine incorporation seen in cultured liver fragments. These data indicate that something in the process of enhanced transcriptional activity and/or vitellogenin induction is responsible for the subsequent changes in mRNA stability. Additional support for this concept comes from experiments with the transcriptional inhibitor actinomycin D. Pretreatment of liver cubes with actinomycin D prevents the degradation of albumin mRNA by estrogen (J.E.M., unpublished results). However, actinomycin D alone stabilizes albumin mRNA complicating interpretation of these results. The factor responsible for destabilization of the serum protein-coding mRNAs could be another RNA, perhaps an RNA cofactor of an enzyme as in the case of RNase P (Pace and Smith, 1990) or a ribozyme itself. Although experiments with the in vitro assay system described in Chapter V suggest the nuclease activity to be protein, experiments are in progress using micrococcal nuclease to address this issue. Another possibility is that a limited pool of a pre-existing RNA-binding protein, such as PABP, whose presence in a complex with mRNA is necessary for stability. As transcription increases, the requirement for PABP increases, but the PABP pool size does not increase. The large accumulation of vitellogenin mRNA itself after estrogen-treatment could sequester this limited pool by mass action and/or greater affinity such that other serum protein-coding mRNAs are more vulnerable for degradation.

Chapter IV: Localization of Albumin mRNA Degradation

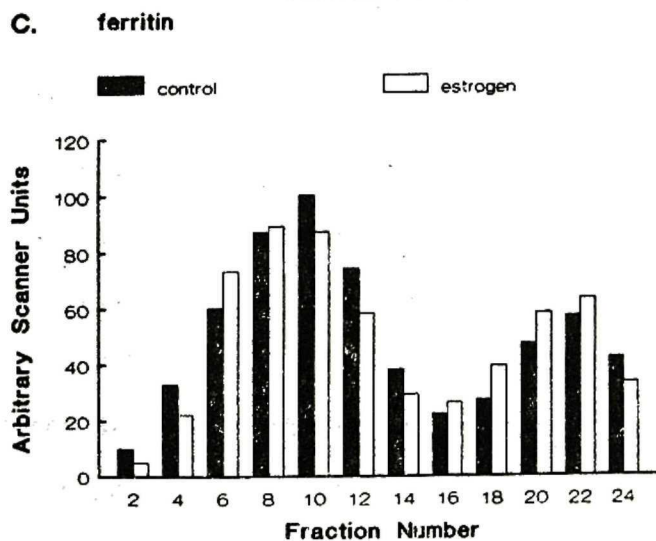
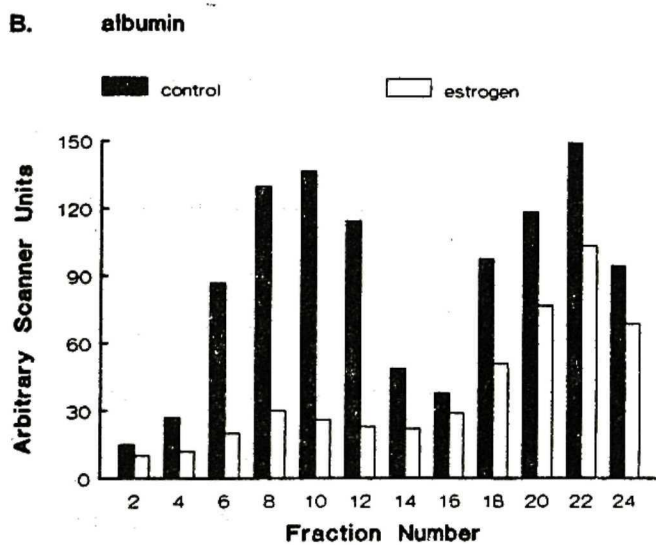
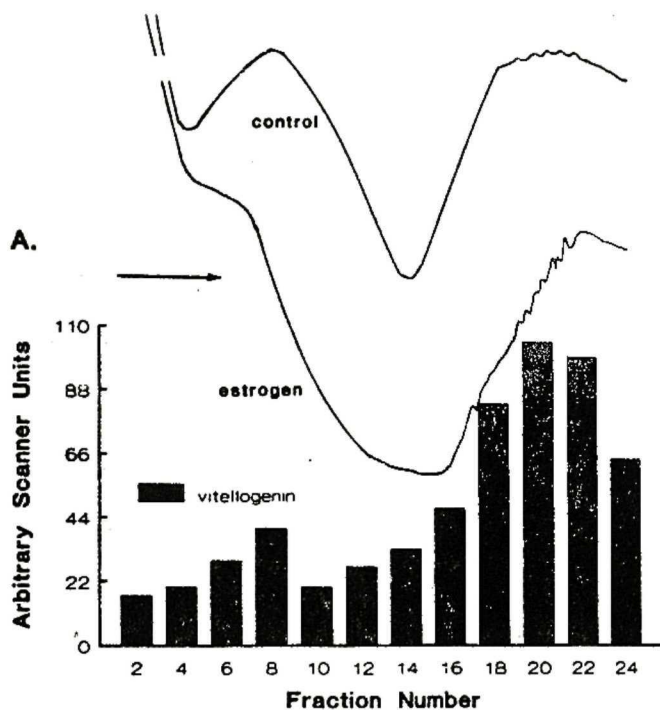
Previous studies from Dr. Schoenberg's laboratory has shown that administration of a single 1 mg dose of estradiol to male Xenopus resulted in the loss of 90% of cytoplasmic albumin mRNA present in the liver (Schoenberg et al., 1989). This occurs by 12 hr after estrogen administration and nuclear albumin mRNA levels are unaffected (Schoenberg et al., 1989). Albumin mRNA stability is significantly decreased without concomitant alterations in albumin gene transcription (Riegel et al., 1986; Kazmaier et al., 1985; Wolffe et al., 1985). Identification of the subcellular site at which albumin mRNA is destabilized is crucial to understanding the mechanism of post-transcriptional regulation of albumin gene expression by estrogen. In the cytoplasm, albumin mRNA is complexed to proteins in the form of mRNP particles (20-80S), ribosomal subunits, or polyribosomes (>80S). Determining the distribution of albumin mRNA in extracts from control or estrogen-treated animals will aid in the understanding of its estrogen-regulated destabilization.

RESULTS

The subcellular location of the cytoplasmic albumin mRNA degradation was characterized using post-mitochondrial extracts (including polysomes and cytosol) prepared from the livers of control and estrogen-treated male Xenopus. The association of albumin mRNA with subcellular particles was examined by sedimentation of the particles through linear 15-40% sucrose density gradients. Figure 17 shows the effect of estrogen treatment on the relative amounts and distribution of mRNP particles, free ribosomes and polysomes upon gradient sedimentation. In control animals the amount of material with absorbance at 260

Figure 17. Sedimentation Profiles and mRNA Distributions of Post-Mitochondrial Extracts of Control and Estrogen-Treated Xenopus Liver.

Male Xenopus were injected 48 hours prior to sacrifice with a single 1 mg injection of estradiol (estrogen) or vehicle alone (control). Post-mitochondrial extracts from each (equal OD₂₆₀ units) was applied to a linear 15-40% sucrose gradient containing a 70% sucrose cushion and centrifuged at 225,000 x g_{max} for 3.5 hours at 4°C. Twenty-four fractions of 0.45 ml each were collected on ice. RNA was extracted from each fraction and assayed for vitellogenin (A), albumin (B), and ferritin (C) mRNA as described in Materials and Methods. Data are plotted as the mean amount of each RNA present in pairs of adjacent fractions. The profiles of material absorbing at 260 nm obtained during the fractionation procedure are superimposed on the pattern for vitellogenin mRNA distribution shown in panel A. The data was quantified by scanning laser densitometry and shown in tabular form in Table III. The direction of sedimentation is shown by the arrow in panel A.



nm is almost equivalent between the 20-80S particle fraction, and the polysome fraction. There is a significant change in this profile 48 hr after estrogen administration, with a decrease in the relative amount of material in the 20-80S portion of the gradient and an increase in polysomal material. Furthermore, the relative position of the polysomal material is shifted to greater density, indicating increased ribosome binding on translating mRNAs. The change in sedimentation profiles can be seen by 24 hr after estrogen-treatment and upon increased time of hormone treatment the shift in albumin mRNA towards heavier polyribosomes becomes more evident.

The relative amounts and distribution of albumin, ferritin and vitellogenin mRNA were determined by dot blot analysis of RNA extracted from each fraction of the gradient. The data quantified by scanning laser densitometry are presented in Figure 17 and shown in tabular form in Table III. In liver from control animals albumin mRNA is equally distributed between the 20-80S mRNP fraction and the polysomal fraction. However, estrogen administration results in a change in albumin mRNA distribution on the gradient, with the majority (80%) of the surviving albumin mRNA (the amount of which is 15-30% of that found in the control) present in polysomes. The steady-state levels of ferritin mRNA are unaffected by estrogen-treatment (Figures 6, 7). Ferritin mRNA is found predominately in the 20-80S mRNP fraction and its distribution between this fraction and the polysome fraction is unaffected by estrogen, despite the significant overall change in the gradient profile (as seen in the A_{260} plot). As expected, the newly induced vitellogenin mRNA is found predominately in the polysome or actively translating fraction of the gradient. Sedimentation analysis of post-mitochondrial extracts from cultured liver cubes exposed to estradiol in vitro yield patterns for RNP, polysome and mRNA distribution identical to those observed in Figure 17.

Table III: Distribution of mRNA Levels for Albumin, Ferritin, and Vitellogenin in Control and Estrogen-Treated Xenopus laevis.

	Control		Estrogen (24 hr)	
	<u>mRNPs</u>	<u>Polysomes</u>	<u>mRNPs</u>	<u>Polysomes</u>
Albumin	50±7	50±7	6±3	24±4
Ferritin	64±8	36±6	51±6	34±7
Vitellogenin	0	0	25±9	75±10

Post-mitochondrial extracts were prepared from control and estrogen-treated animals and sedimented through 15-40% sucrose gradients to separate mRNPs (20-80S) from polyribosomes (>80S). The relative amounts and distribution of albumin, ferritin, and vitellogenin mRNA were determined by dot blot analysis of RNA extracted from each fraction of the gradient. Data are presented as mRNA distributions in fractions from 20-80S (mRNP) sedimenting material and >80S (polysomes) sedimenting material. The data was quantified by scanning laser densitometry. Data represent the mean ± standard error from five separate experiments. Albumin mRNA is distributed equally in mRNP and polysome fractions from control animals. Upon estrogen-treatment only 30% of albumin mRNA remains (compared to control) and 80% of this remaining mRNA is found on polyribosomes and 20% on mRNP particles. Ferritin mRNA is distributed such that 64% of the message is in mRNPs and 36% in polysomes from control extracts. Upon estrogen treatment, of the 85% ferritin mRNA remaining, 60% is in mRNPs and 40% in polysomes. Vitellogenin mRNA levels are shown as a positive control. Vitellogenin mRNA is mainly distributed in polysomes. This value increased upon longer treatment with estradiol.

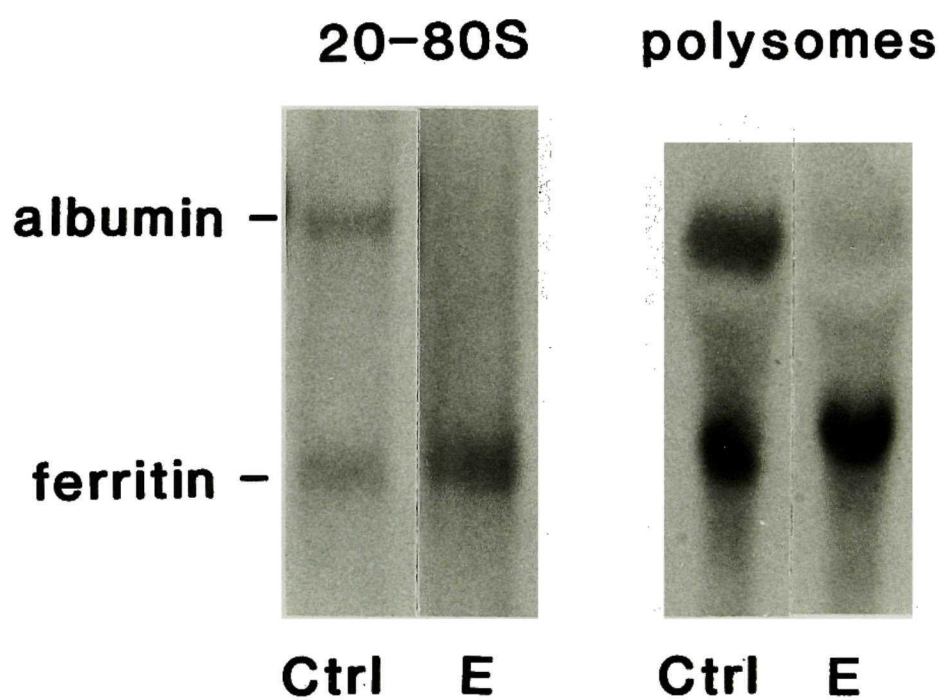
The preferential disappearance of albumin mRNA on mRNPs observed by dot blot analysis of individual fractions was confirmed by the Northern blots shown in Figure 18. Samples corresponding to the 20-80S portion of the gradient (mRNP) were pooled as were samples corresponding to polysomes (>80S). Pooled fractions were electrophoresed on a 1% agarose gel and the resultant blot was hybridized with a mixture of albumin and ferritin cDNAs. Albumin mRNA was undetectable in mRNP fractions from estrogen-treated animals whereas the amount of ferritin mRNA remained unchanged. Estrogen administration resulted in a decrease in the amount of albumin mRNA present in polysome fractions, however the decrease was less than that observed in the mRNP fractions. Ferritin mRNA levels in the polysome fraction remained unaffected by hormone treatment. It is surprising that the distribution of ferritin mRNA remains unchanged after estrogen treatment in spite of the substantial change in A_{260} profile and its well-characterized translational regulation. The data in Figures 17 and 18 indicate that cytoplasmic albumin mRNA disappears preferentially from the 20-80S mRNP fractions after treatment of Xenopus with estradiol.

DISCUSSION

Estrogen administration to male Xenopus laevis or to liver explants from the same animals caused a generalized disappearance of mRNAs encoding serum proteins from the cytoplasm. To localize further the site of albumin mRNA degradation in the cytoplasm after estrogen-treatment, post-mitochondrial extracts were fractionated on sucrose gradients. These results suggested that albumin was being degraded before reaching the polyribosomes as 20-80S mRNP fractions contained 20% of the remaining albumin mRNA after estrogen-treatment (Figure 17, Table III). Albumin mRNA is equally distributed in mRNP and

Figure 18. Northern Blot Analysis of Polysomes and mRNPs from Post-Mitochondrial Extracts of Nonestrogenized or Estrogenized Xenopus.

Samples corresponding to the 20-80S portion of the 15-40% sucrose gradient (mRNP) and the polysomes were each pooled, phenol/chloroform extracted and the RNA precipitated with ethanol. Samples were glyoxalated, electrophoresed on a 1% agarose gel and the resultant blot was hybridized with a mixture of radiolabeled albumin and ferritin cDNAs. Extracts were prepared from animals that received either vehicle (Ctrl) or 1 mg of estradiol (E) 48 hr prior to sacrifice.



polyribosome fractions in extracts from control animals. In contrast, the distribution of ferritin mRNA, which is relatively unaffected by estrogen, was unchanged between the two fractions (Figure 17, Table III). About 60% of ferritin mRNA is distributed on mRNPs and 40% distributed on polyribosomes in extracts from either control or estrogen-treated animals. Vitellogenin mRNA was seen mainly distributed on heavier polyribosomes only in extracts from estrogen-treated animals (Figure 17, Table III) as previously reported (Blume and Shapiro, 1989). Northern blot analysis of pooled mRNP and pooled polyribosome fractions from control and estrogen-treated animals confirmed that there is less albumin mRNA in the mRNP fraction upon estrogen-treatment when ferritin mRNA levels were only slightly affected (Figure 18).

The different mechanisms of action of the translation inhibitors used in Chapter III further support the mRNPs as the subcellular site of albumin mRNA destabilization. Sucrose gradient analysis of RNA-protein complexes present in the post-mitochondrial supernatant of Xenopus cultured liver cubes exposed to cycloheximide or MDMP confirmed that these reagents resulted in the redistribution of polysomal material (Figure 14). The inhibitors were therefore used to augment (cycloheximide) or disrupt (MDMP) the formation of polysome complexes after initiation of albumin mRNA destabilization by estradiol. If binding to polysomes was important for the regulation of albumin mRNA one would have expected cycloheximide to potentiate, and MDMP to inhibit the disappearance of albumin mRNA. Instead, both inhibitors had no effect on mRNA destabilization.

The most likely site for the destabilization of albumin mRNA is on mRNP complexes present in the 20-80S sedimenting material. Albumin mRNA preferentially disappeared from 20-80S sedimenting material by 24 hr after estrogen treatment. It is possible that most of albumin mRNA is being degraded on mRNP particles and the remaining albumin mRNA is then further degraded on

polyribosomes. In fact, using an in vitro assay system to study mRNA degradation as described in Chapter V, nuclease activity can be found in fractions of liver extracts sedimenting at >80S (polyribosomes), and to a smaller extent in 20-80S sedimenting fractions (mRNP particles). Recently, Bandyopadhyay et al. (1990) have demonstrated that a nuclease activity believed to be important in the regulation of mammalian mRNA stability is present in tight association with mRNA in both polysome and mRNP fractions. It is possible this nuclease could transfer between polysomes and mRNP particles as mRNA is transferred to actively translating polysomes. It is also possible there is a block in reinitiation of the polyribosomes and albumin mRNA is prevented from binding. The remaining mRNA on the polyribosomes could be that which was previously bound and the released material could be degraded. To address these possibilities an in vitro assay system has been developed and is described in Chapter V.

The data are consistent with the hypothesis that mRNP complexes are important in differentiating albumin and other serum protein-coding mRNAs from ferritin mRNA in the degradation process. It will be important now to analyze the mRNP complexes for albumin and ferritin to see what factors in each complex may allow ferritin to be more resistant to degradation.

Chapter V: Development of an in vitro Assay System to Study mRNA Stability

To investigate cis elements and trans-acting factors which are potentially important in regulating albumin and other mRNA turnover, an in vitro nuclease assay for studying mRNA degradation was developed. This chapter describes the nuclease isolation and assay conditions and successfully demonstrates that the differential stability observed after estrogen treatment between albumin and ferritin mRNA in vivo can be reproduced in vitro.

RESULTS

Figure 19 lists the conditions that were selected to study mRNA degradation in the in vitro assay system and a schematic of fractions which were tested for nuclease activity. Post-mitochondrial extracts were fractionated on 15-40% linear sucrose gradients containing a 70% sucrose cushion to separate messenger ribonucleoprotein particles (mRNPs) from heavier polyribosomes (Figure 20). These fractions were pooled and tested for their ability to degrade endogenous albumin mRNA under in vitro assay conditions (Figure 20). Nuclease activity was found to be greatest in polyribosome fractions (lanes 6, 7) and less in mRNP fractions (lanes 4, 5) or in the unfractionated post-mitochondrial extract (lanes 1, 2, 3) which was the source of the material fractionated on the gradient. The small amount of nuclease activity in post-mitochondrial extracts observed upon incubations for as long as 60 min suggests the presence of an inhibitor in the extract which fractionates upon gradient centrifugation. For this reason, polyribosomes were chosen as a source of nuclease activity in subsequent assays. The effect of estrogen-treatment on nuclease activity found in the polyribosomes was then examined (Figure 21). Total liver RNA as substrate was incubated for 30 min at 22°C with equal amounts of polysomes (based on A_{280} nm) from animals that received vehicle alone (E-) (lanes 2, 3) or 1 mg estradiol (E+) (lanes 4, 5). The polysomes served as the source of nuclease. Surviving RNA was analyzed by Northern blot. Control (lane 1) represents total RNA incubated without any added polysomes. There was increased nuclease activity (on a per μ g basis) found in polysomes from estrogen-treated Xenopus compared to untreated animals (lanes 4, 5 vs. lanes 2, 3). Activity increased 4-10 fold based on three separate experiments. Figure 22 shows that nuclease activity can be removed from polyribosomes of estrogen-treated animals. Polyribosomes were

Figure 19. In vitro Cell-Free Assay Conditions for Studying mRNA Turnover and Schematic of Characterization of Nuclease Activity.

A. Reaction conditions to study mRNA degradation are listed. After incubation, substrates were treated with 0.5% SDS, 100 μ g proteinase K for 1 hr at 42°C. A synthetic [32 P] labeled tracer RNA (truncated antisense albumin transcript) was added, standard phenol/chloroform extractions were performed and RNA precipitated by ethanol. Northern blot analysis was then performed to determine the quality and quantity of RNA remaining.

B. Schematic of the characterization of nuclease activity is shown. Post-mitochondrial extracts were prepared in which nuclei, mitochondria, and lysosomes were removed by sequential centrifugations. The resulting supernatant was centrifuged and the pellet containing heavier particles (>80S) was fractionated on sucrose gradients. The fractions corresponding to >80S particles (polysomes) contained nuclease activity. This activity could be extracted from the polysomes with 0.4 M NaCl and could be further purified by salt elution from a phosphocellulose column. Activity was also found in polysome fractions from post-mitochondrial extracts directly fractionated on 15-40% sucrose gradients (labeled PELLET (Ribosomes)).

A

IN VITRO CELL-FREE ASSAY SYSTEM TO STUDY mRNA TURNOVER:

SUBSTRATE (TOTAL LIVER RNA, 32P-transcripts, mRNPs, Polyribosomes)

30 mM Tris-HCl, pH 7.4

2 mM Mg⁺⁺

1 mM ATP

+ / - Fraction of Extract presumed to contain activity

Incubate for various times at 22°C.

SDS / Proteinase K treatment, add tracer.

Phenol / Chloroform extractions.

Northern blot analysis to determine quality and quantity of RNA.

B

SCHEMATIC OF CHARACTERIZATION OF NUCLEASE ACTIVITY

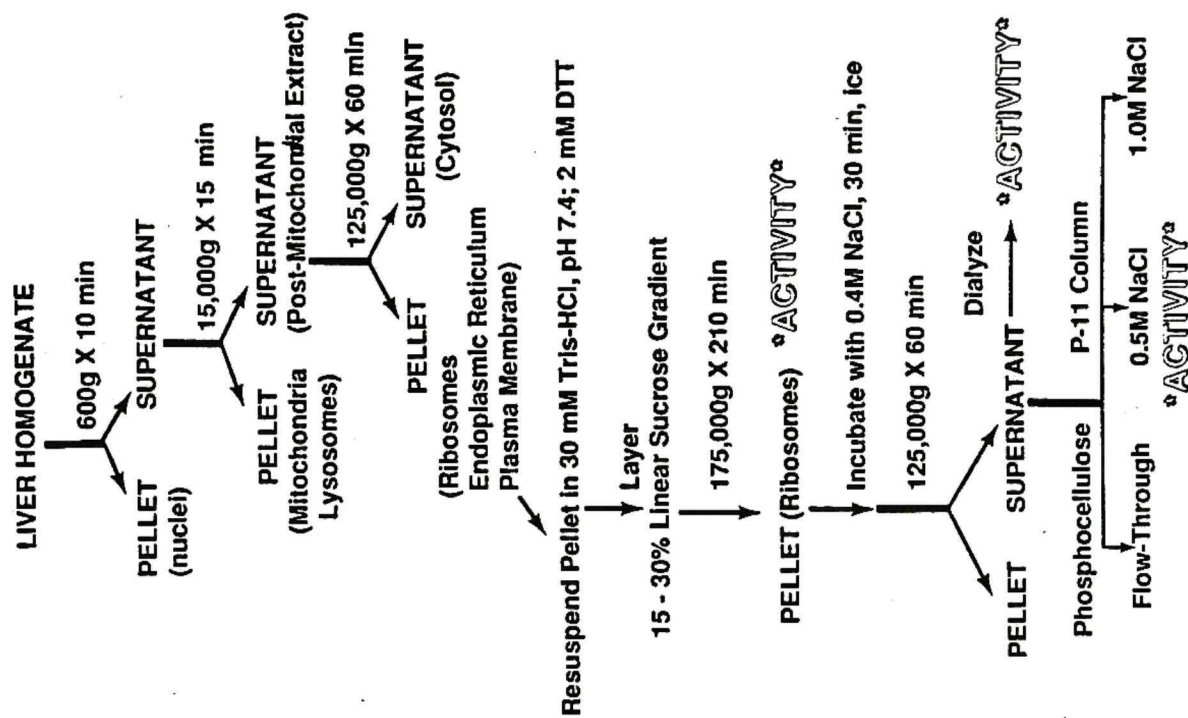


Figure 20. Localization of Nuclease Activity to Polyribosomes.

Post-mitochondrial extracts were fractionated on 15-40% sucrose gradients to separate 20-80S particles (messenger ribonucleoprotein particles, mRNPs) from heavier polyribosomes (>80S). These fractions were tested for their ability to degrade endogenous albumin mRNA under in vitro assay conditions (Figure 19A). Lanes 1-3 are the remaining RNA from the unfractionated post-mitochondrial extract incubated for 30 or 60 min at 0°C or 22°C. Lanes 4 and 5 are 20-80S particles from the sucrose gradient incubated for 60 min at 0°C or 22°C. Lanes 6 and 7 are polysomes from the sucrose gradient incubated for 60 min at 0°C or 22°C. Equal A_{260} units were incubated in lanes 1, 2 and 3; lanes 4 and 5; and in lanes 6 and 7. Albumin mRNA levels were quantified by laser scanning densitometry and are represented as a percent of material incubated for 60 min at 0°C, lanes 1, 4 or 6 (100%).

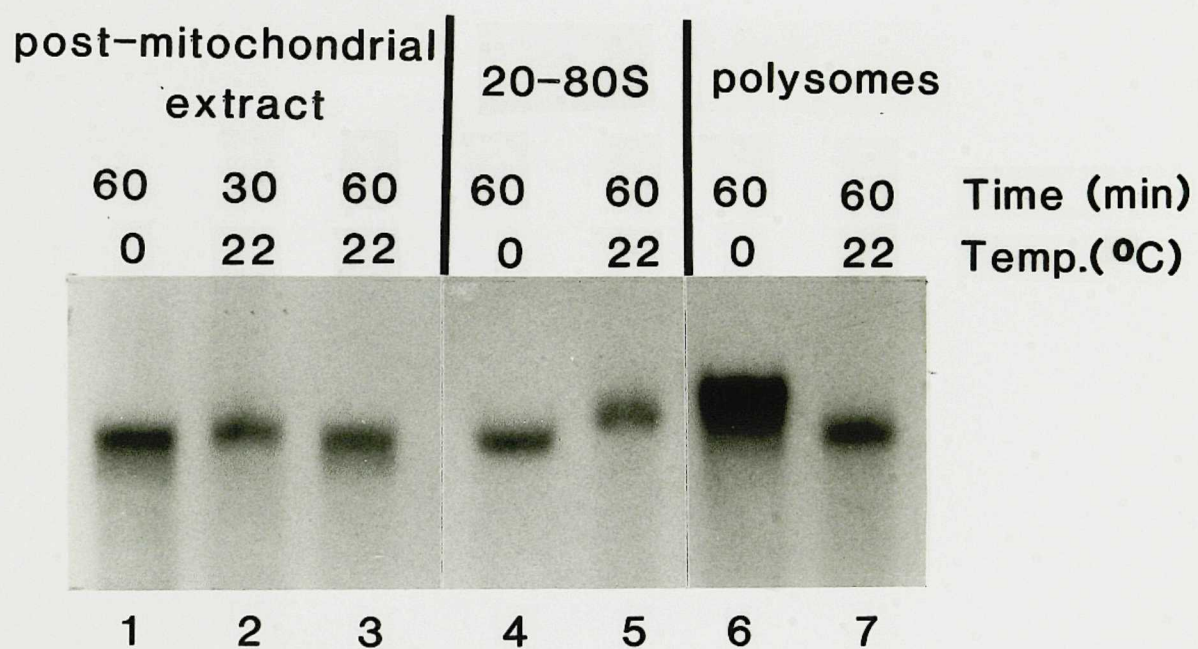


Figure 21. Effect of Estrogen-Treatment on Nuclease Activity.

Ten micrograms of total liver RNA as substrate was incubated for 30 min at 22°C with equal amounts (based on A_{260} units) of polysomes (enzyme source) from animals that received vehicle alone (- polysomes, lanes 2 and 3) or 1 mg estradiol for 24 hr (+ polysomes, lanes 4 and 5) under in vitro assay conditions. RNA was extracted and Northern blot analysis performed. Lane 1 represents total RNA incubated without any added polysomes. The signal seen is that for albumin mRNA.

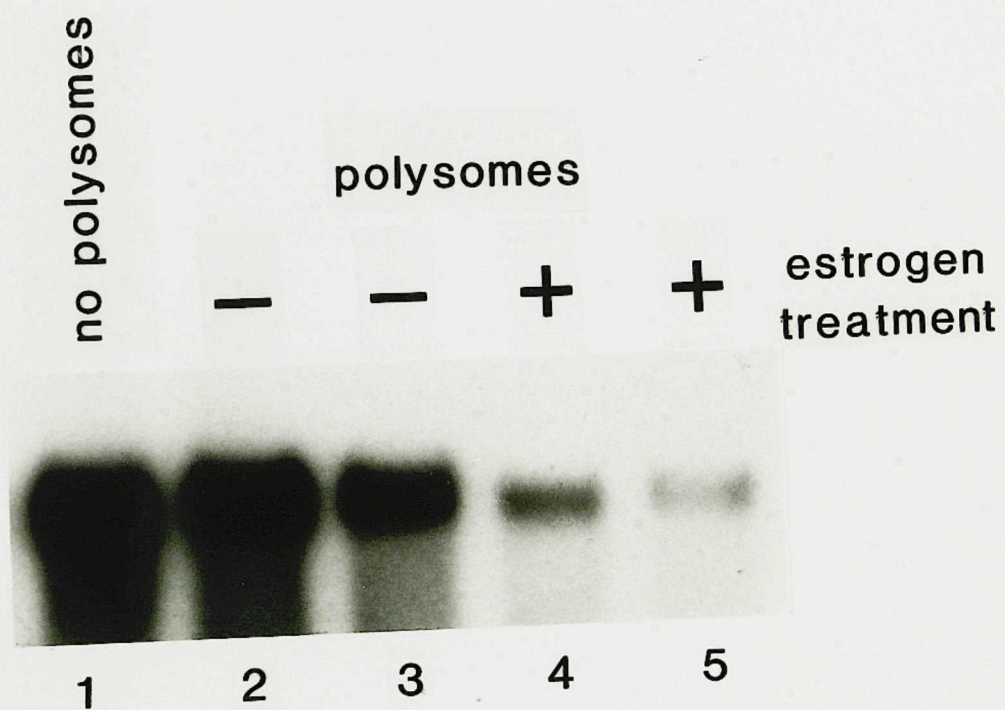
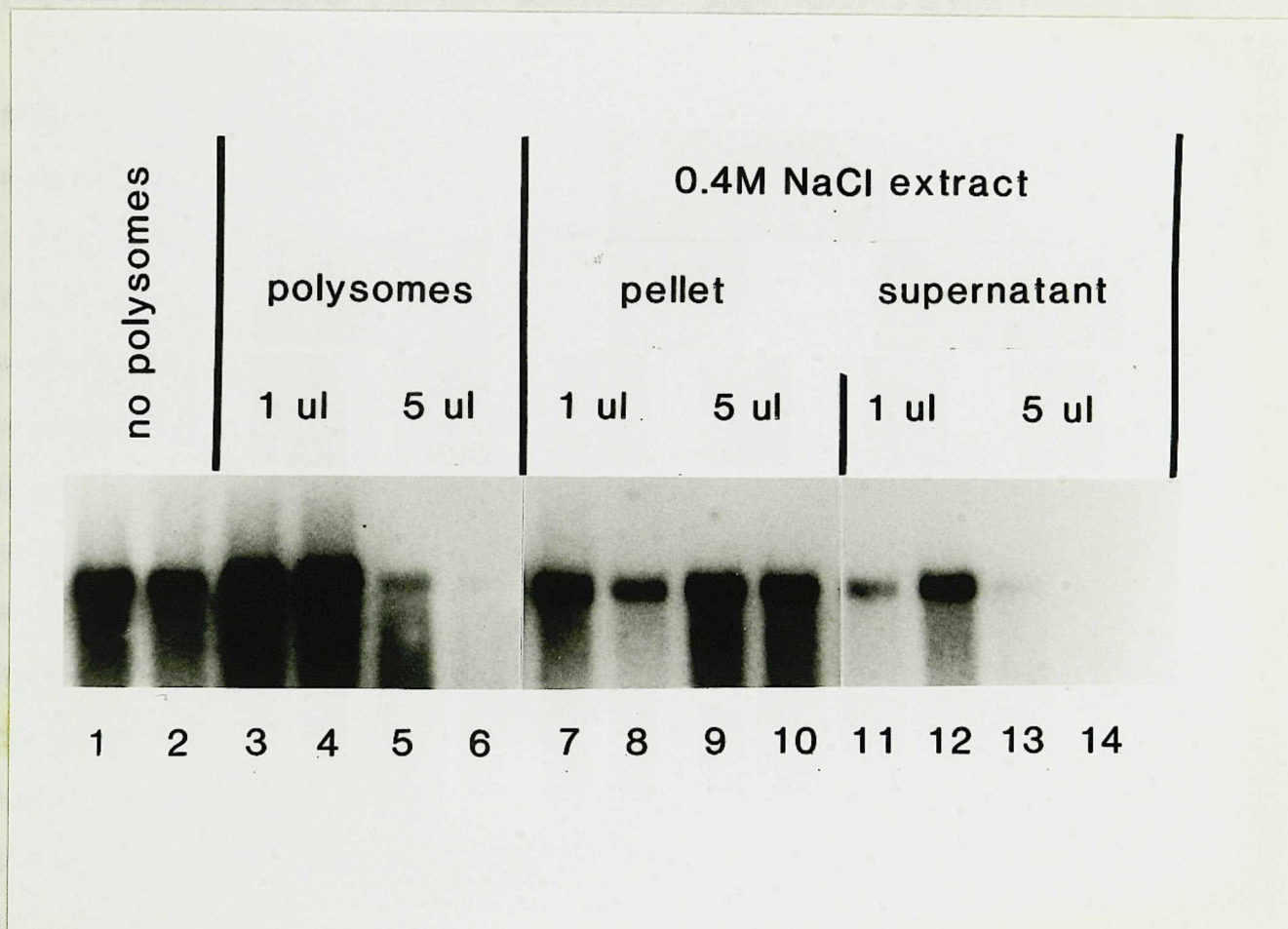


Figure 22. Extraction of Nuclease Activity from Polyribosomes.

Ten micrograms of total liver RNA as substrate was incubated with polysomes (enzyme source) from animals that received 1 mg estradiol for 24 hr. (lanes 3, 4 = 1 μ l polysomes; lanes 5, 6 = 5 μ l polysomes) or these same polysomes that had been salt extracted (lanes 7, 8 = 1 μ l pellet after extraction; lanes 9, 10 = 5 μ l pellet after extraction; lanes 11, 12 = 1 μ l supernatant after extraction; lanes 13, 14 = 5 μ l supernatant from salt-extracted polysomes). All reactions were incubated under the same in vitro assay conditions (total volume = 30 μ l). Lanes 1 and 2 represent incubation of substrate without polysomes. RNA was extracted and Northern blot analysis performed. Signal seen represents albumin mRNA.



treated with 0.4 M NaCl and the resultant extract and polyribosomes were tested for their ability to degrade albumin mRNA. Total RNA was utilized as the substrate during incubations with these fractions and Northern blot analysis was performed afterwards. Nuclease activity was quantitatively extracted by 0.4 M NaCl. The high-salt supernatant contained nuclease activity (lanes 11-14) comparable to the initial polysome fraction (lanes 3-6) but the salt-extracted polysome pellet (lanes 7-10) did not contain appreciable activity.

Figures 23-25 indicate differential in vitro degradation of albumin and ferritin mRNAs using either total RNA (Figure 23), [^{32}P]labeled transcripts synthesized in vitro (Figure 24), or messenger ribonucleoprotein particles (mRNPs) (Figure 25) as substrate. Total RNA (5-20 μg) (Figure 23) was incubated for 30 min with 1 or 5 μl of supernatant of salt-extracted polysomes from untreated (lanes 3, 4, 7) or estrogen-treated (lanes 5, 6, 8, 9) animals (equal A_{280} units). In Figure 24 [^{32}P]labeled albumin and ferritin transcripts (50 ng) were incubated with 1 and 3 μl of supernatant of salt-extracted polysomes from estrogen-treated animals for 15, 30 and 45 min (lanes 3-8). mRNPs (Figure 25) were incubated with the supernatant of salt-extracted polysomes (lanes 3 & 4) or without polysomes (lanes 1 & 2). The supernatant of salt-extracted polysomes was the source of nuclease activity. A tracer consisting of an [^{32}P]-labeled truncated anti-sense albumin transcript was added to the reaction mixture after incubation to control for extraction efficiency. Using either total RNA (Figure 23), [^{32}P]transcripts (Figure 24), or mRNP particles (Figure 25) as substrates, the nuclease present in extracts from polysomes of estrogen-treated animals degraded albumin mRNA faster than ferritin mRNA. This differential activity is similar to that demonstrated in vivo and in cultured liver cubes (Figure 7). Fibrinogen and transferrin mRNAs, which are regulated in vivo like albumin are also degraded more rapidly than ferritin.

Figure 23. Differential in vitro Degradation of Albumin and Ferritin mRNAs using Total RNA as Substrate.

Ten micrograms of total RNA substrate was incubated for 30 min under in vitro assay conditions with 1 or 5 μ l of salt-extracted polysomes (containing nuclease activity- see Figure 25) from untreated (-) or estrogen-treated (24 hr (+)) animals. Polysomes from untreated or estrogen-treated animals had equal optical density units at 280 nm. Lanes 1 and 2, no polysomes added; lanes 3 and 4, 1 μ l polysomes from untreated animals; lanes 5 and 6, 1 μ l polysomes from estrogen-treated animals; lane 7, 5 μ l polysomes from untreated animals; lanes 8 and 9, 5 μ l polysomes from estrogen-treated animals; lanes 1 and 2, no polysomes added. Northern blot analysis was performed and albumin and ferritin mRNA signals are shown.

* represents a tracer (400 nucleotide anti-sense albumin transcript) added to reaction mixture after incubation to insure equal extraction of RNA from each reaction. RNA levels were quantified by laser scanning densitometry and shown below. Solid bar represents albumin mRNA levels; open bar represents ferritin mRNA levels. Values in the bar graph are relative to incubation without any added polysomes (lanes 1 and 2) and normalized to the tracer (*).

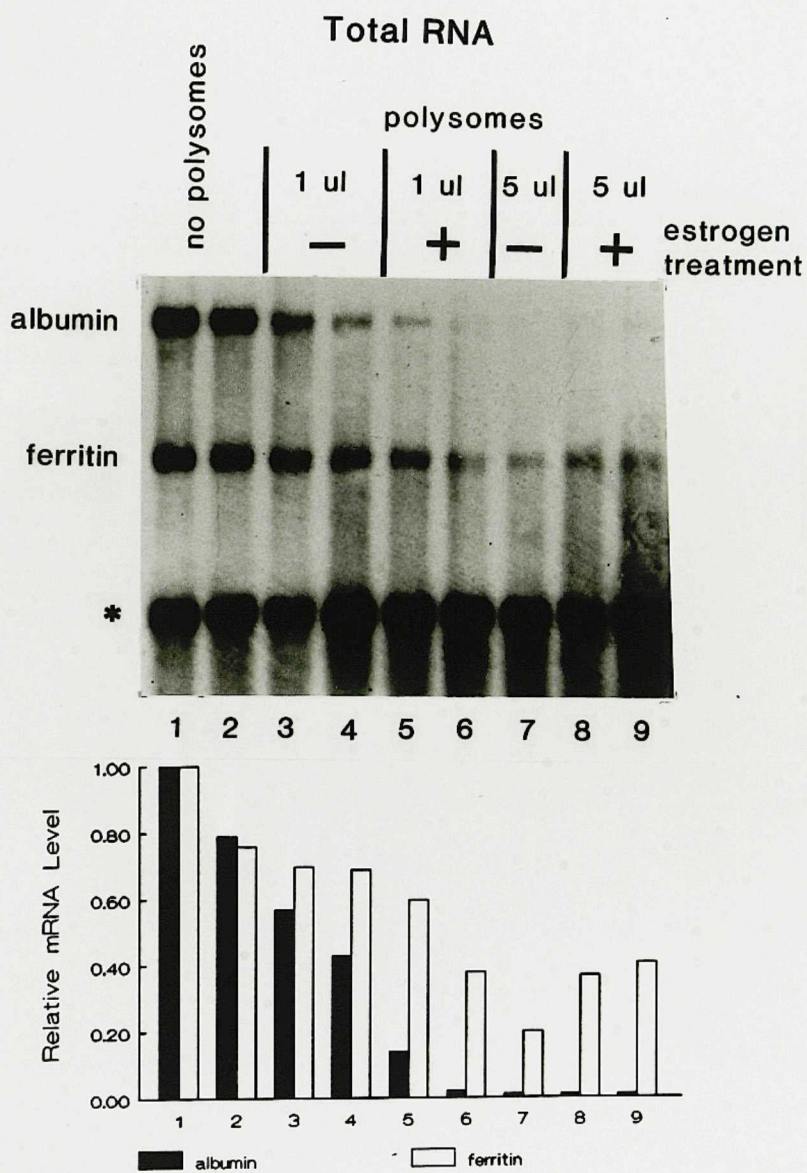


Figure 24. Differential in vitro Degradation of Albumin and Ferritin mRNAs using [^{32}P]transcripts as Substrate.

Fifty nanograms of [^{32}P] labeled full length albumin and ferritin transcripts synthesized in vitro were incubated with polysomes from estrogen-treated (24 hr) animals for 15 min (lanes 3 and 6), 30 min (lanes 4 and 7), or 45 min (lanes 5 and 8) under in vitro assay conditions. RNA was extracted, glyoxalated, electrophoresed on an 1% agarose gel. The gel was then dried, and autoradiographed. Signals for albumin, ferritin and the tracer * are shown. Tracer (anti-sense albumin transcript) was added to the reaction after incubation to insure equal extraction of RNA from each reaction. Lanes 1, 2, and 9 represent incubation without any added polysomes. Messenger RNA levels were quantified by laser scanning densitometry of autoradiographs and normalized to the tracer (shown below). RNA levels are relative to the incubations without any added polysomes (1.00).

-- O -- albumin mRNA levels; -- • -- ferritin mRNA levels.

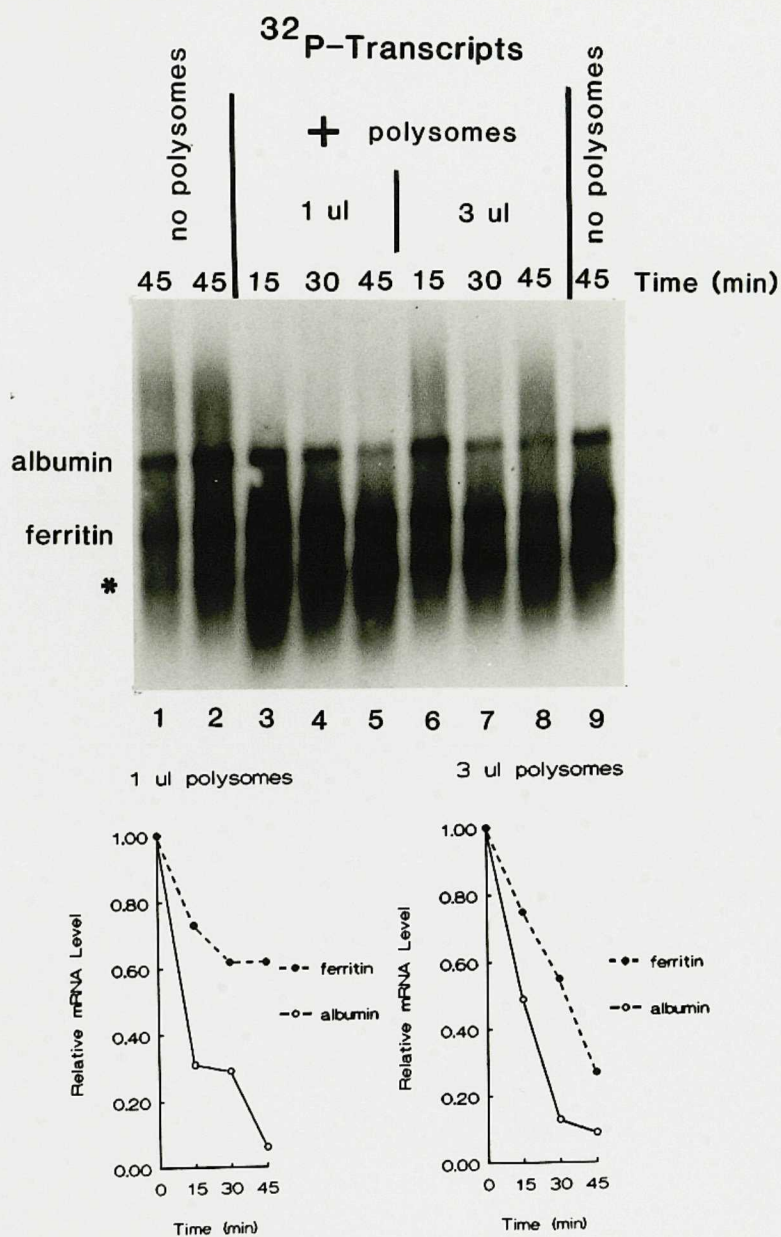
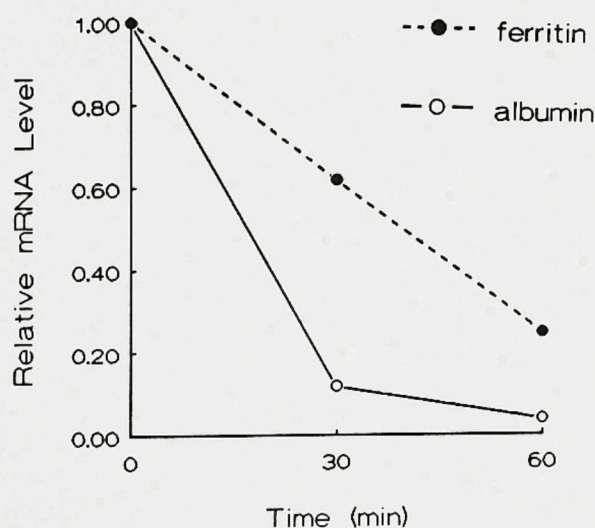
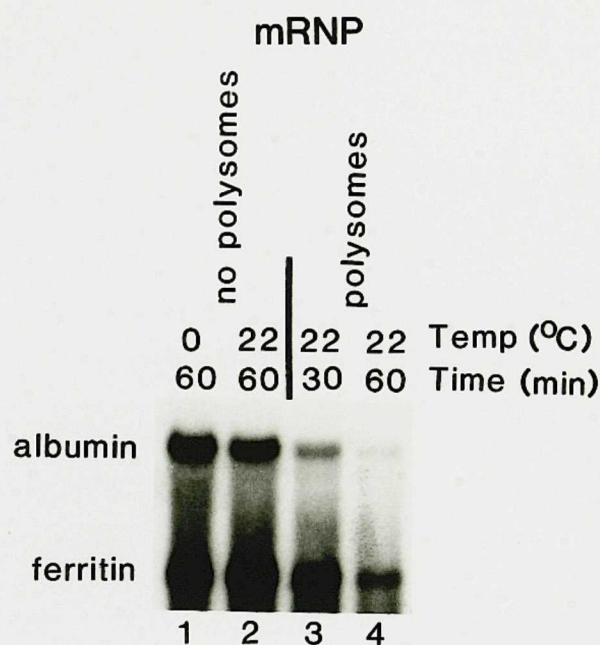


Figure 25. Differential in vitro Degradation of Albumin and Ferritin mRNAs using Messenger Ribonucleoprotein Particles (mRNPs) as Substrate.

Messenger ribonucleoprotein particles from control animals were used as substrate and incubated with supernatant from salt-extracted polysomes (source of nuclease activity) from estrogen-treated (24 hr) animals for 30 min and 60 min at 22°C. RNA was extracted and precipitated with ethanol and Northern blot analysis performed. Albumin and ferritin mRNA levels were quantified by laser scanning densitometry (below) relative to incubation without any added polysomes (lanes 1 and 2).

-- O -- albumin mRNA levels, -- • -- ferritin mRNA levels.

The nuclease activity present in the supernatant of polyribosomes was further purified by chromatography on phosphocellulose. Figure 2f shows the activity of the purified nuclease at various salt concentrations. The activity was found only in the 0.5 M NaCl eluted material.



The nuclease activity present in the supernatant of salt-extracted polyribosomes was further purified by chromatography on phosphocellulose. Figure 26 shows the activity of nuclease present in fractions eluted with increasing salt concentrations. Substantial activity was found only in the 0.5 M NaCl eluted material from the column (lanes 7, 8) but not in the flow-through (lanes 5, 6) or 1.0 M NaCl (lanes 9, 10) fractions. This step allows a partial purification of nuclease activity.

The nuclease behaves as a single activity under non-denaturing gel electrophoresis (Figure 27). Fifty μ l of a 0.5 M NaCl extract of polysomes was electrophoresed on a 7% polyacrylamide nondenaturing gel and two millimeter slices were cut from the top to the bottom of the gel. Each slice was then incubated for 45 min under in vitro assay conditions in the presence of albumin and ferritin transcripts. After incubation, RNA was extracted from each reaction and Northern blot analysis performed. A nuclease activity which degrades albumin transcript more rapidly than ferritin transcript is seen in slices 2-4. Only one peak of activity is seen (slices 2-4) throughout the gel suggesting a single nuclease activity.

The nuclease activity was further characterized by testing its requirements for divalent metal ions, ATP, NaCl, pH, temperature, and sensitivities to proteinase K and RNase inhibitors. Table IV lists the various effects of each variable on nuclease activity. Magnesium and ATP at concentrations up to 20 mM had no effect on nuclease activity. EDTA at concentrations of 0-40 mM was added to the reaction mixture and did not abolish activity suggesting that divalent cations are not important for activity. Sodium chloride was also added to the reaction mixture and at concentrations up to 200 mM did not have an effect on activity but at greater concentrations the activity was inhibited as much as 75%.

Figure 26. Nuclease Activity Localized to 0.5 M NaCl Eluted Fraction off Phosphocellulose Column.

A 0.5 M NaCl extract from polyribosomes of estrogen-treated animals was chromatographed on a phosphocellulose P-11 column and bound proteins were eluted with increasing salt concentrations (flow-through, lanes 5 and 6; 0.5 M NaCl, lanes 7 and 8; 1.0 M NaCl, lanes 9 and 10). These fractions were incubated with 10 μ g total liver RNA as substrate under in vitro assay conditions. Lanes 3 and 4, total RNA incubated with salt-extracted polysomes; lanes 1, 2, 11, and 12, incubation without any added polysomes. RNA was extracted, precipitated with ethanol and Northern blot analysis performed. * represents tracer (truncated anti-sense albumin transcript) added after incubation to insure equal extraction of RNA from each reaction.

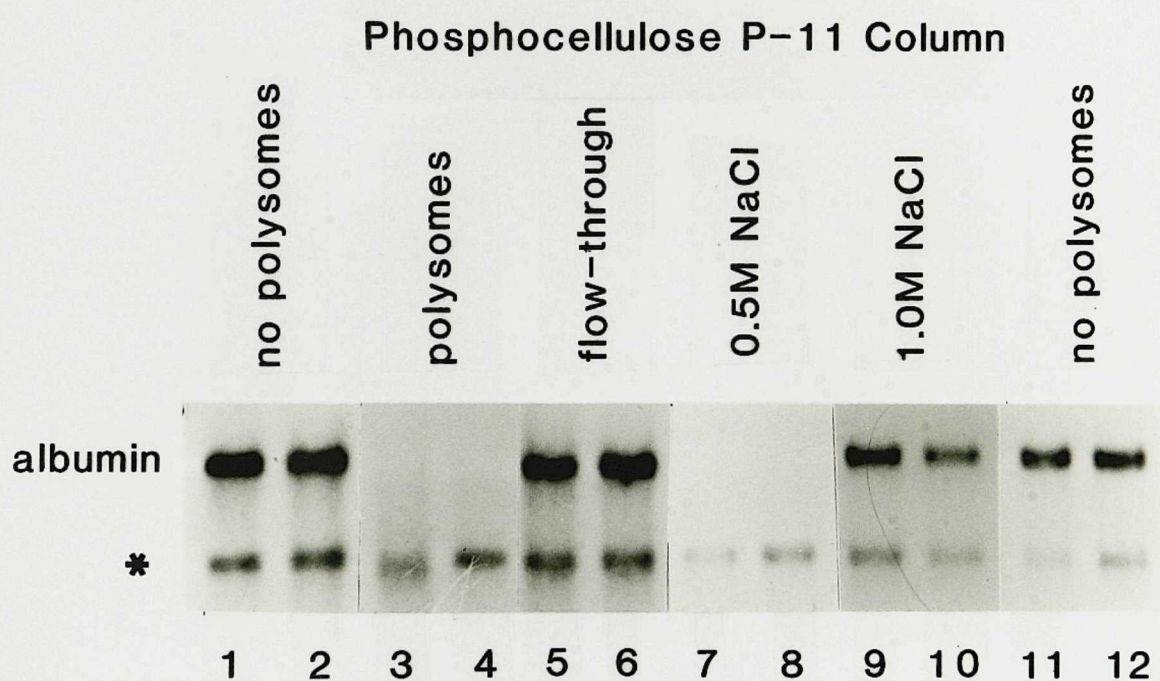


Figure 27. Measurement of Nuclease Activity in Non-Denaturing Gels.

Fifty microliters of the supernatant from salt-extracted polyribosomes were electrophoresed on a 7% acrylamide nondenaturing gel and 2 mm slices were cut from top (lane 1) to bottom (lane 14). Each slice was incubated for 45 min under in vitro assay conditions in the presence of albumin and ferritin transcripts. RNA was extracted and Northern blot analysis performed. Albumin and ferritin mRNA levels for each reaction (slice) were quantified by laser scanning densitometry and represented as a ratio of albumin to ferritin.

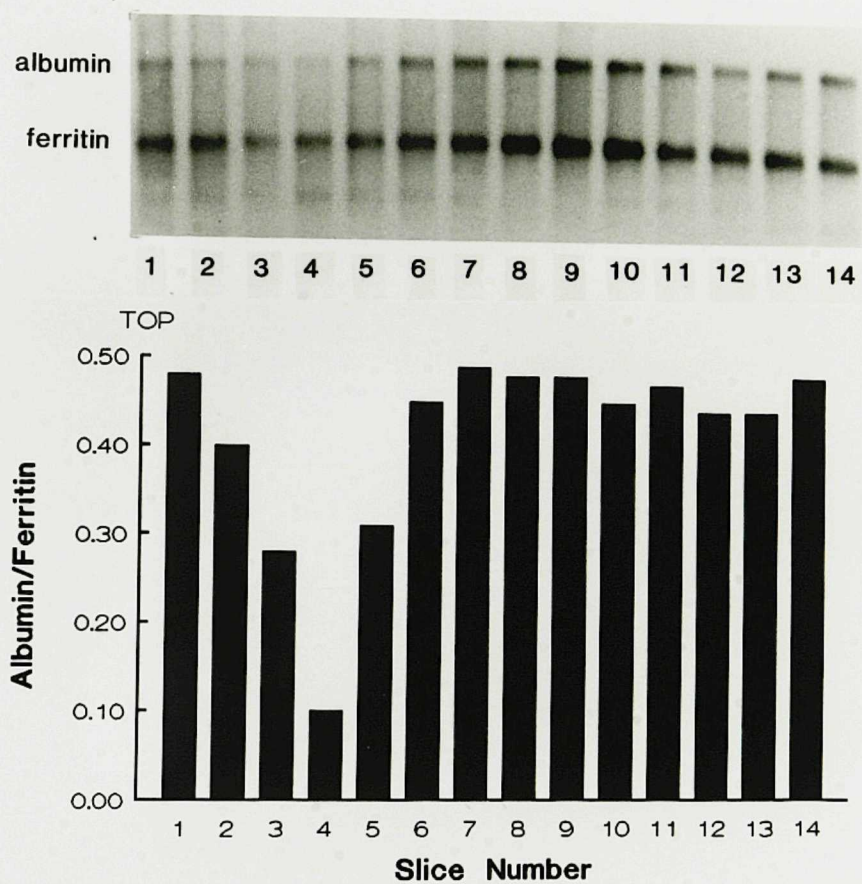


Table IV: Characterization of Nuclease Activity

<u>Factor</u>	<u>Requirement</u>
ATP	-
Mg ⁺⁺	-
+ EDTA	-
pH	+ , optimum pH = 7.8
Temperature	+ , optimum T _{rxn} = 37°C; preincubation of extract optimal T = 22°C
+ tRNA	+ , competes
NaCl	+ , < 200 mM for activity
RNasin	+ , inhibits activity
Inhibit-Ace	+ , inhibits activity
Proteinase K	+ , inhibits activity

Various factors were tested for their ability to affect nuclease activity. -

, no effect on activity; + factor had an effect on activity.

The pH of the in vitro reaction was varied from 6.0 to 8.8. Optimal nuclease activity was achieved at pH 7.8. High temperature had adverse effects on the nuclease activity. The temperature of the in vitro reaction was varied from 0°C to 90°C. The greatest nuclease activity occurred at a reaction temperature of 37°C, although at 22°C, 95% of maximal activity was present. In addition to varying the in vitro reaction temperature, the supernatant of 0.4 M salt-extracted polyribosomes containing the nuclease activity was preincubated at 0°C, 22°C, 37°C, 65°C, and 90°C for 1 hr. The incubated extracts were then tested to contain nuclease activity under standard assay conditions (22°C). Under these conditions, maximal activity was found when the extract was preincubated at 22°C. Ninety percent of activity remained after preincubating the extract at 37°C, 50% of activity after preincubation at 65°C and <10% of activity after preincubation at 90°C. Incubating the supernatant of the salt-extracted polyribosomes with proteinase K/SDS abolished the activity, suggesting that the activity resides in a protein. Also, nuclease activity could be abolished by titrating with standard ribonuclease inhibitors (RNasin and Inhibit-Ace). The activity was inhibited by addition of increasing amounts of substrate or addition of greater than 10 µg tRNA to the reaction, which presumably act as competitors for the RNA binding site.

DISCUSSION

An in vitro assay system was developed to study estrogen-regulated mRNA degradation in Xenopus laevis. Nuclease activity was identified in polyribosome fractions. This activity could be extracted with salt and further purified on a phosphocellulose column. Nuclease activity was eluted from the column with 0.5 M NaCl. The amount of nuclease activity found in the polysome fraction was hormone regulated as polysomes from estrogen-treated animals contained greater activity

than corresponding fractions from control-treated animals on an equal protein basis.

Nuclease activity was minimal in crude post-mitochondrial extracts but after fractionation greater nuclease activity was found in polyribosome fractions. This suggests the presence of a ribonuclease inhibitor in the post-mitochondrial extract that could be separated from the polyribosomes during fractionation on sucrose gradients.

The function of RNase inhibitors and their mode of interaction with RNases are relatively unknown although it appears that RNase inhibitors may be an important controlling element in RNA metabolism and protein synthesis (Gagnon and Lamirande, 1973; Zan-Kowalczevska and Roth, 1975; McGregor et al., 1981; Penttila and Maenpaa, 1985). RNase inhibitors have been found in mammalian, plant, and bacterial cells and prevent the degradation of nuclear RNA (Hymer and Kuff, 1964), as well as mRNA associated with polyribosomes (Blobel and Potter, 1966; Lawford et al., 1967; Grau and Favelukes, 1968). Ribonuclease inhibitor levels have been found to correlate with changes in cell growth and proliferation (Brewer et al., 1969; Moriyama et al., 1969; Kraff and Shortman, 1970; Little and Meyer, 1970).

A ribonuclease inhibitor in the uterine cytoplasm of rats (McGregor et al., 1981) and rooster liver ribosomes (Penttila and Maenpaa, 1985) are responsive to estrogen. In both cases, ribonuclease activity increases and ribonuclease inhibitor becomes undetectable during estrogen-induced RNA and protein synthesis. A change in the ratio of ribonuclease inhibitor to ribonuclease upon estrogen-treatment could lead to altered mRNA stability and these possibilities are currently being addressed.

The nuclease activity found in polyribosomes was increased upon estrogen-treatment. This is the first demonstration of a hormone-regulated nuclease

activity that appears to mediate the differential stabilities of hormone-regulated and nonregulated mRNAs. Experiments using protein synthesis inhibitors in cultured liver cubes have shown that new protein synthesis is not required for the estrogen-regulated destabilization of albumin and other serum-protein-coding mRNAs. Yet, in vitro data demonstrate an increased nuclease activity found in polyribosomes from estrogen-treated animals compared to control. These experiments at first glance appear to contradict one another. Inhibitor experiments with cycloheximide and MDMP suggest that albumin mRNA destabilization is a primary effect of hormone while in vitro data suggest the increase in nuclease activity after estrogen-treatment is most likely secondary to the destabilization. The reaction protocols in both sets of experiments are different. In vitro assay experiments used post-mitochondrial extracts prepared 24-72 hr after estrogen-treatment of Xenopus. These data could be explained by increased synthesis or activation of a pre-existing nuclease after hormone treatment. A post-translational modification could be responsible for activation. Estrogen has been shown to cause post-translational modifications of proteins including phosphorylation/dephosphorylation (Haffar et al., 1987).

In Chapter IV, the possible importance of the mRNP complex in differentiating serum protein-coding mRNAs and intracellular mRNAs for destabilization was suggested. Phosphorylation of mRNP proteins has been documented (Slegers et al., 1981; Rittschof and Trangh, 1982; Deardy et al., 1985; Kick et al., 1987). This group includes the helix-destabilizing protein (HD40) from Artemia salina (Slegers et al., 1981), and a 60 KDa mRNP protein from Xenopus oocytes (Kick et al., 1987). Two-dimensional polyacrylamide gel electrophoresis resolves poly(A) binding proteins into several posttranslationally modified forms (Drawbridge et al., 1990). Phosphorylation of PABP has been suggested to be a likely modification involved in regulating mRNA

stability (Drawbridge et al., 1990) and could play a role in estrogen-regulated destabilization of mRNAs as previously described. Estrogen-treatment could increase the activity of a preexisting factor by changing the conformation or affinity of the factor with the mRNA leading to altered stability of the mRNA. A ribozyme could also be responsible for the degradation of serum protein-coding mRNAs.

The nuclease activity present in the supernatant of salt-extracted polyribosomes shows a selective degradation of the estrogen-regulated mRNAs as has been described in vivo or in cultured liver cubes. The rank order of mRNA decay rates in liver cells after estrogen treatment is reflected under in vitro assay conditions. Albumin and fibrinogen mRNAs are degraded rapidly in cells and in vitro whereas ferritin mRNA is relatively stable in both. The kinetics of transferrin and clone 12B mRNA degradation were also examined in the in vitro assay system and found to be degraded with similar kinetics to albumin mRNA as was seen in vivo (Figure 8). Experiments are in progress using truncated transcripts and chimaeric mRNA constructs to determine the recognition site(s) of the nuclease on the regulated mRNAs.

All three substrates examined (total RNA, [³²P]transcripts, and mRNP particles) showed a differential rate of degradation between albumin and ferritin mRNAs. The [³²P] labeled transcript data suggests that the mRNA primary structure itself is important in differentiating these messages for degradation as the naked transcripts were not complexed with proteins as they are in vivo. The greatest difference in the rate of degradation between albumin and ferritin mRNAs was seen with the mRNP particles. This argues that the mRNA-protein complex influencing secondary structure is also important and may allow the ferritin complex to be more resistant to the degradative factor(s) than the albumin or other serum protein mRNP complexes. Conversely, mRNP structure may

make albumin mRNA more susceptible to degradation. These data along with the translation inhibitor data that support the mRNP complex as the site of the cytoplasmic degradation, leads one to speculate that the mRNP complexes are the important distinguishing factor determining the rate of degradation. Degradation of mRNA could also be occurring on polyribosomes. This is supported from in vitro experiments in which polyribosomes from estrogen-treated animals contain nuclease activity.

Other in vitro systems have been described to study mRNA degradation (Ross and Kobs, 1986; Ross et al., 1987; Peltz et al., 1987; Brewer and Ross, 1989; Pei and Calame, 1988; Sunitha and Slobin, 1987; Bandyopadhyay et al., 1990). Nuclease activity has been isolated from polyribosomes (Ross and Kobs, 1986; Pei and Calame, 1988; Bandyopadhyay et al., 1990) as well as mRNPs (Sunitha and Slobin, 1987; Bandyopadhyay et al., 1990). Extraction with high salt removed activity from polysomes or mRNPs in many of these systems.

The nuclease activity was characterized and found to be independent of divalent cations or ATP for activity. There are many cases in the literature of RNases which are dependent on (Ross et al., 1987; Pei and Calame, 1988) as well as independent of divalent ions (Sunitha and Slobin, 1987). They also demonstrate various energy requirements. The effect of magnesium on nuclease activity was also determined. Magnesium can modify the structure of RNA and therefore can alter ribonuclease activity. Magnesium at concentrations up to 20 mM had no effect on nuclease activity. The nuclease had a pH optimum of 7.8. Temperature also affected nuclease activity. There was much less ribonuclease activity at temperatures of 65°C, and 90°C than at more physiological temperatures.

The ribonuclease activity appears to be protein since incubation of the polysome extract with proteinase K/SDS completely inhibited activity.

Ribonuclease inhibitors (RNasin, Inhibit-Ace) also blocked activity. RNasin ribonuclease inhibitor has been shown to inhibit the activity of RNase A-type enzymes in a variety of organisms (Blackburn et al., 1977). Inhibit-Ace is a nonspecific ribonuclease inhibitor. The nuclease that degrades histone mRNA is active in the presence of RNasin (Ross and Kobs, 1986). Preliminary experiments have shown that the nuclease activity extracted from polysomes is endonucleolytic and experiments are in progress to map sensitive cleavage sites on the message.

SUMMARY AND CONCLUSIONS

The phenomenon of estrogen-induced mRNA degradation in Xenopus hepatocytes is not restricted to albumin. γ -fibrinogen, transferrin, the second protein of inter- α -trypsin inhibitor, and an unidentified serum protein mRNA termed 12B were all found to be destabilized by estrogen. To date, all of the mRNAs found to be destabilized by estrogen encode secreted proteins. Other mRNAs encoding secreted proteins such as vitellogenin and retinol binding protein are stabilized in these same cells after treatment with estradiol. One can conclude that the majority of proteins secreted by the frog liver are degraded by a post-transcriptional mechanism since the albumins, fibrinogens, and transferrin together comprise greater than 85% of liver secreted proteins. Other mRNAs encoding intracellular proteins such as actin, ferritin, reference clone pXref-1, poly(A)-binding protein and globin were unaffected by estrogen-treatment.

The first question addressed was whether the serum protein coding mRNAs destabilized after estrogen-treatment shared any common sequence or structural features that may target their degradation. Both albumins, γ -fibrinogen,

transferrin and portions of the second protein of inter- α -trypsin inhibitor and an unidentified serum protein mRNA termed 12B were sequenced and there are no sequences conserved among these mRNAs. The albumins and γ -fibrinogen do share highly homologous sequences in the 3'-untranslated region near the poly(A) addition site. However, the other estrogen-regulated mRNAs did not share these same sequences. The importance of this conserved sequence and whether one or more mechanisms may be involved in the regulation of serum protein coding mRNAs by estradiol have yet to be demonstrated. However, no gross sequence or structural feature that could be recognized by a degradative factor is present which would explain the coordinate regulation seen.

One feature found to be characteristic of serum protein coding mRNAs destabilized by estradiol was a short poly(A) tail. Both albumins, γ -fibrinogen and transferrin mRNAs had poly(A) tails ranging in size from 16 to 30 residues in length. The length of the poly(A) tail for these mRNAs did not change upon estrogen administration. Therefore, poly(A) shortening cannot be considered to be the primary mechanism responsible for the changes in the rate of degradation of these mRNAs. Since the assays used only measured the degree of steady-state polyadenylation, it is possible that the serum protein-coding mRNAs are extensively polyadenylated followed by a rapid removal of poly(A).

In contrast, vitellogenin mRNA which is stabilized by estrogen has a long poly(A) tail ranging from 35-215 residues. A reference clone (pXref-1), the stability of which is unaffected by estrogen, has a poly(A) tail greater than 200 residues. Ferritin mRNA (also unaffected by estrogen) has a poly(A) tail 40-60 residues in length, similar to that of the stable β -globin mRNA (Merkel *et al.*, 1975). Therefore, not all liver mRNAs possess short poly(A) tails and the relationship between poly(A) tail length and mRNA destabilization is unclear.

The complex formed between poly(A) and the poly(A)-binding protein (PABP) has been shown to be important for the formation of a translation initiation complex (Sachs and Davis, 1989). One hypothesis consistent with the data presented here is that albumin, fibrinogen and transferrin mRNA may be poised to be destabilized by virtue of their short poly(A) tail. The minimum poly(A) length for stable binding of PABP is 12 residues (Sachs et al., 1987). The short poly(A) tails on the serum protein-coding mRNAs are sufficient to bind only a single molecule of PABP. In contrast, vitellogenin mRNA can bind 8-9 molecules of PABP (based on a packing density of one molecule of PABP per 25 adenosine residues, Sachs et al., 1987).

There is a reciprocal relationship between the disappearance of albumin mRNA and the induction of vitellogenin mRNA. The induction of vitellogenin mRNA might result in competition for initiation complex formation (perhaps mediated by PABP) resulting in enhanced degradation of serum protein coding mRNAs after release from polysome complexes upon translation termination or prior to translation initiation on newly synthesized serum protein-coding mRNA. Since these mRNAs can barely bind a single molecule of PABP, and PABP expression is unaffected by estrogen, it is conceivable that the induction of vitellogenin mRNA is enough to change the balance between protein-bound and unbound species so that the PABP-deprived mRNAs with shorter poly(A) tails become more susceptible to nucleolytic attack. This concept has been proposed for c-myc and c-fos mRNAs in which AU-rich instability sequences in the 3'-untranslated regions of these mRNAs could cause redistribution of PABP rendering the mRNA more susceptible to degradation (Bernstein et al., 1989; Brewer and Ross, 1985; Wilson and Treisman, 1988). It remains to be determined whether the amount of PABP is limiting in Xenopus hepatocytes or whether trans-acting factors present in the liver direct the addition of only a short 3' poly(A). Transferrin mRNA present in the frog

oviduct, like that in the male frog liver, has a short poly(A) tail but upon estrogen-treatment (which results in a significant induction of transferrin mRNA) the length of the poly(A) tail increases significantly (Pastori et al., in preparation). Experiments are in progress to address the nature of the regulation of polyadenylation in these tissues.

Sucrose gradient analysis of cytoplasmic RNA-protein complexes extracted from livers of control and estrogen-treated animals indicate that albumin mRNA disappears preferentially from the 20-80S peak of mRNP particles, ribosomal subunits and free monosomes. Ferritin mRNA, whose steady-state levels are unaffected by estrogen, showed no change in distribution between the 20-80S peak and the polysome fraction, even though estrogen causes a significant shift in the amount of material which absorbs at 260 nm from the 20-80S fractions to heavier polysome fractions.

The different mechanisms of action of cycloheximide and MDMP enabled further characterization of the subcellular site of albumin mRNA destabilization. If binding of mRNA to polysomes was important for the regulation of albumin one would have expected cycloheximide to potentiate, and MDMP to inhibit the degradation of albumin mRNA. Instead, both inhibitors at concentrations which blocked protein synthesis by 90-95% had no effect on the estrogen-mediated disappearance of albumin mRNA. This confirmed that the most likely site for the cytoplasmic degradation is the mRNP complexes present in the 20-80S sedimenting material. This is in contrast to tubulin (Yen et al., 1988), *c-fos* (Wilson and Treisman, 1988), histone (Graves et al., 1987) and vitellogenin (Blume and Shapiro, 1989) mRNAs whose relative stability or instability involves either polysome binding or active translation. It is possible that some albumin mRNA is also degraded on polysomes as supported by in vitro data.

The importance of the mRNP complex in differentiating the various mRNA species opens a wide variety of projects. The biochemical analysis of mRNP complexes from stable (vitellogenin), unaffected (ferritin), and destabilized (majority of serum proteins) mRNAs can indicate the importance of proteins involved in each complex. In addition, reconstitution of the mRNP complexes can also be performed. The further characterization of the ribonucleoprotein particles containing the specific proteins involved in the regulation of mRNA stability and their eventual cloning will shed considerable light on the role of protein-nucleic acid interaction in the regulation of RNA stability.

An in vitro assay system to study estrogen-regulated mRNA stability was developed as part of this project. This was the first such in vitro assay system to study RNA stability that was hormonally regulated. Activity was found in polyribosomes from animals treated with estradiol. Protein synthesis was not required for the estrogen-regulated destabilization of albumin mRNA yet greater activity was found in polyribosomes from estrogen-treated animals. Estrogen could cause a post-translational modification of an existing protein or change the balance of the ratio of ribonuclease/ribonuclease inhibitor. It is also possible that an RNA transcribed in response to estradiol is involved in the degradation of a number of serum protein-coding mRNAs. Experiments are in progress to address these possibilities.

It is unclear why ferritin mRNA levels remain relatively unaffected by estrogen. Ferritin mRNA encodes an intracellular protein, hence it is likely to be translated on free rather than membrane bound polyribosomes, and it may also form different mRNP complexes than the serum protein-coding mRNAs. Ferritin has a longer poly(A) tail than the serum protein-coding mRNAs. Xenopus ferritin has a long leader (at least 294 nucleotides) containing the palindromic iron response element (Moskaitis et al., 1990). There may be some unique structural feature to

ferritin mRNA which distinguishes it from the others, as the naked transcript is degraded much more slowly than serum protein-coding mRNAs in the in vitro mRNA degradation system.

The agents that degrade mRNA and hence control mRNA turnover have not been well characterized in eukaryotic systems. Cytoplasmic ribonucleases and other factors that bind and degrade mRNA molecules will need to be examined. Which of these factors degrade mRNAs? Which factors degrade other types of RNA molecules such as rRNA and tRNAs? Are these factors the same or are they different? Do certain mRNA degrading ribonucleases act only on specific mRNAs? Do several different ribonucleases act together to degrade individual mRNAs? These are some of the questions that can begin to be answered using the in vitro system described in this thesis.

The long term objectives of this research are to identify the specific molecules involved in the post-transcriptional destabilization of mRNA through further analysis with the in vitro system and to use these as tools to selectively introduce this form of regulation into the expression of any gene of interest. The factors involved in hormone regulated mRNA stability can be compared to factors described in the handful of other in vitro systems.

The discoveries that stem from this research provide fundamental understanding of cellular mechanisms regulating mRNA metabolism. It is hoped that in vitro assay systems to study mRNA stability could be utilized in examining the factors involved in a wide variety of disease states in which the rate of mRNA turnover is important.

APPENDIX

The following are additions to the Materials and Methods section.

During the start of my dissertation work (Summer, 1986) I set out to develop a method for sequencing double-stranded DNA directly. This would be useful as labor-intensive subcloning could be avoided. This proved successful and the work was presented at the USUHS Graduate Student Colloquium in April of 1988. Many laboratories during this time also were developing this methodology and this work has been published elsewhere. Below is a description of various aspects of double-stranded DNA sequencing. The effort to perfect this method proved useful to my dissertation as seen in the many sequence figures presented within. A great many ideas and suggestions from many laboratories including ours has led to the latest protocol (now considered routine) described by United States Biochemical Corp., the producer of Sequenasetm. Just 2-3 years ago this methodology was in its infancy.

DNA Sequencing Reaction Protocol

All sequencing reactions were performed in 0.5 ml plastic centrifuge tubes. These were capped to minimize evaporation of the small volumes employed. Additions were made with disposable-tip micropipettes on the side wall of the centrifuge tubes. Care should be taken not to contaminate stock solutions and to avoid the creation of air bubbles. The solutions were mixed after addition of reagents by pulse centrifuging for seconds.

A wide variety of conditions for sequencing supercoiled plasmid DNA were investigated including template isolation, denaturation of duplex DNA, annealing of primer to template, sequencing reaction conditions (temperature, DNA template/primer molar ratios and amount of enzyme used) and gel electrophoresis conditions. Three enzymes were utilized to catalyze these reactions: 1) the Klenow fragment of *E. coli* DNA polymerase I; 2) AMV (avian myeloblastosis virus) reverse transcriptase; and 3) a modified (exonuclease deficient) T7 DNA polymerase (Sequenasetm, T7DNAP). Autoradiographic resolution was best using high quality template DNA (free of RNA, proteins, or salts), ³⁵S-labelled dATP, gradient electrophoresis gels using wedge spacers and 1.0X Tris-borate buffer, and T7DNAP. The quality of DNA polymerization using T7DNAP was greater than that using either Klenow or AMV reverse transcriptase. Artifactual banding patterns due to masking bands seen across all four lanes of a gel and autoradiographic background was present to a greater extent in Klenow and AMV reverse transcriptase DNA polymerized samples. Using T7DNAP, DNA sequencing gels have uniformly labelled bands with reduced background allowing the resolution of more bases in a single experiment. DNA sequencing reaction conditions were optimized for T7DNAP. The following is a description of optimal conditions used for plasmid DNA sequencing. Figure 28 lists a brief summary of the sequencing protocol using T7DNAP.

Alkali Denaturation

The alkaline-denaturation method (Chen and Seeburg, 1985) gives high quality, reproducible results. Four to six μ g of plasmid DNA was denatured in 0.4 M NaOH for 5 min at room temperature. The mixture was neutralized by adding 0.2 volumes of 3 M sodium acetate, pH 5.5 and the DNA precipitated with

Figure 28. Protocol for Sequencing Double-Stranded DNA using T7 DNA Polymerase (Sequenasetm).

Summary of steps involved in sequencing plasmid DNA using a modified (exonuclease deficient) T7 DNA polymerase (Sequenasetm). A detailed description of each step is described in the Appendix.

T7 DNA POL. (SEQUENASE™)

ALKALI DENATURATION
OF PLASMID DNA TEMPLATE



NEUTRALIZATION AND
PRECIPITATION OF DNA



ANNEALING OF DENATURED
DNA TEMPLATE AND PRIMER



DNA POLYMERIZATION REACTION

a. Labelling Reaction

dNTPs

3 U T7 DNA Polymerase, DTT

[α - ^{35}S]-dATP (5 uCi, 1000-1500 Ci/mmol)

Incubate at 37°C, 5 min

b. Termination Reaction

ddNTPs

Incubate at 37°C, 5 min



STOP REACTION

Formamide, EDTA



DENATURE AND LOAD

three volumes of ethanol at -70°C , 30 min. After washing the pelleted DNA with 75% ethanol, it was redissolved in 7 μl of distilled water, and 2 μl of T7DNAP buffer and 1 μl (10 ng) of primer added (total volume equals 10 μl).

Annealing Template and Primer

For each set of four sequencing lanes, a single annealing and subsequent labeling reaction was used. A capped centrifuge tube containing the plasmid DNA, sequencing buffer, and primer was warmed to 65°C for 3 min. The temperature of the tube was allowed to cool slowly to room temperature over a period of 30 min. Cooling was done by using a heating block placed at room temperature on the bench. Once the temperature was below 35°C , annealing was complete. For the annealing reaction, a 5-fold molar excess of template DNA over primer was found to be optimal.

Labeling Reactions

For standard reactions (reading sequences up to 400 bases from the primer), the dGTP labeling mix (7.5 μM dGTP, 7.5 μM dCTP, 7.5 μM dTTP) was diluted 5-fold with distilled water. For sequencing within 30 bases of the primer, dilution was 15-fold. The amount of template DNA and primer must both be greater than 0.5 pmol. Insufficient DNA or primer will reduce the labeling of the first few nucleotides from the primer. The T7DNAP enzyme was diluted 1:4 in ice-cold Tris-EDTA buffer. Only enough enzyme for immediate use was diluted (2 μl per reaction). Six units of T7DNAP was found to be optimal in sequencing plasmid DNA. Diluted enzyme was stored on ice for no longer than 60 min. To the annealed DNA template-primer (10 μl), 1 μl of 0.1 M dithiothreitol, 2 μl of diluted labeling mix, 5 μCi of $[\alpha\text{-}^{35}\text{S}]$ dATP (1000-1500 Ci/mmol) and 2 μl (6 units) of diluted T7DNAP were added. The reaction was mixed thoroughly and incubated for 5 min at room temperature. The amount of

labeling mix and radiolabeled nucleotide was adjusted according to the needs of the experiment. For obtaining sequences where band compressions were a problem, the dITP labeling mix (15 μ M dITP, 7.5 μ M dCTP, 7.5 μ M dTTP) was substituted for the dGTP mix. Similarly, the dITP termination mixtures were used in the termination reactions (see below).

Termination Reactions

Four tubes were labeled A, C, G, and T. Next, 2.5 μ l of the ddGTP termination mix (80 μ M dGTP, 80 μ M dATP, 80 μ M dCTP, 80 μ M dTTP, 8 μ M ddGTP, 50 mM NaCl) was placed in the tube labeled G. With a fresh tip for each, the A, T, and C tubes were filled with 2.5 μ l of the ddATP (80 μ M dGTP, 80 μ M dATP, 80 μ M dGTP, 80 μ M dTTP, 8 μ M ddATP, 50 mM NaCl), ddTTP (80 μ M dGTP, 80 μ M dATP, 80 μ M dCTP, 80 μ M dTTP, 8 μ M ddTTP, 50 mM NaCl) and ddCTP (80 μ M dGTP, 80 μ M dATP, 80 μ M dCTP, 80 μ M dTTP, 8 μ M ddCTP, 50 mM NaCl) termination mixtures respectively. The tubes were capped to prevent evaporation. This was best done before beginning the labeling reactions. The tubes were prewarmed at 37°C for 1-3 min. When the labeling reaction incubation was complete, 3.5 μ l was removed and transferred to the tube labeled G. Similarly, 3.5 μ l of the labeling reaction was transferred to the A, T and C tubes. The tubes were mixed, centrifuged and the incubation continued at 37°C for 5 min. Four μ l of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to each of the termination reactions. Tubes were mixed thoroughly and reactions stored on ice until ready to load the sequencing gel. When the gel was ready for loading, the samples were heated to 85°C for 2 min and loaded immediately on the gel. Four μ l was used in each lane.

Alterations of Reaction Conditions

Depending on the region of DNA to be sequenced in a particular experiment, reaction conditions were altered to provide maximum information of sequences close to the primer, or alternatively those more distant from the primer. If the interest was only in sequences close to the primer, the labeling mix was diluted and both labeling and termination reaction times reduced. For reading more distant from the primer, the concentrations of the dNTPs in the labeling reaction were increased five-fold and the labeling reaction time increased to 10 min. The radiolabeled dATP concentration must also be increased. T7DNAP was also used in conjunction with dITP to eliminate compression and other secondary-structure gel artifacts.

Denaturing Gel Electrophoresis

The quality of the gel electrophoresis was a critical factor which limited the extent of sequence information that could be determined in a single sequencing experiment. The length of time the gel was run (2-12 hr) determined the region of sequence that was readable. Electrophoresis grade reagents were used. Acrylamide solutions were made fresh once every two weeks and stored in the dark at 4°C. Gels were prepared 2-4 hours prior to use, and pre-run for 60 min at a constant power of 70 watts. Gels were soaked after running in 10% acetic acid, 10% methanol to remove the urea. Soaking time depended on gel thickness (15 min for 0.4 mm gels to 45 min for 0.2-1.0 mm wedge gels). The drying of the sequencing gel (8 M urea, 4-10% polyacrylamide) was performed at 80°C for typically 1-1.5 hr. Exposure for ³⁵S gels was performed with direct contact between the dried gel and the emulsion side of the film. Overnight to 48 hour exposures were sufficient when using Kodak XAR-5 film. The use of tapered spacers ("wedge" gels)

improved overall resolution and allowed more nucleotide sequence to be read (Ansorge and Labeit, 1984). Sequencing double-stranded templates with T7DNAP was superior to either the Klenow fragment of DNA polymerase I or AMV reverse transcriptase. The T7DNAP sequencing reaction protocol was the simplest, least time consuming, and required the smallest amount of radiolabeled nucleotide, thus allowing multiple samples to be run in a single day (Table V).

Polymerase Chain Reaction for Amplification of 3' end of Albumin cDNA

Amplification of DNA by the polymerase chain reaction was performed as described by Saiki *et al.* (1988) and Oste (1988). Poly(A) RNA and total RNA was prepared from nonestrogenized and estrogen-treated (24 hr) animals and used as templates for first strand cDNA synthesis using oligo(dT) and AMV reverse transcriptase. Incubations were performed at 42°C for 1 hr, additional enzyme added and continued for an additional hour. The mixture was treated with RNase A, phenol/chloroform extracted and the cDNA recovered by ethanol precipitation. This cDNA was used for the polymerase chain reaction (PCR). This method for DNA amplification is based on the repetition of a set of three steps (denaturation of double-stranded DNA; annealing of extension primers; and primer extension-amplification), all conducted in succession under somewhat different and controlled temperature conditions. Oligonucleotides DANS13 corresponding to nucleotides 1783-1799 of the cDNA and oligo(dT) were used as extension primers. Denaturation was performed at 94°C for 1.5 min. Annealing of extension primers was carried out for 2 min at 37°C and the primer extension step for 3 min at 72°C. Amplifications with *Taq* polymerase took place in 100 μ l reaction mixtures containing 0.01-0.5 μ g first strand

TABLE V: SUMMARY OF MAJOR CONSIDERATIONS WHEN SELECTING ENZYME FOR DOUBLE-STRANDED DNA SEQUENCING

<u>SEQUENCING ENZYME:</u>	<u>KLENOW</u>	<u>AMV</u>	<u>SEQUENASE</u>
TIME OF REACTION (MIN)	35	35	10
AMOUNT RADIOLABELLED NUCLEOTIDE (uCi)	40	40	5
MAXIMUM AMOUNT OF SEQUENCE READ (nts)	250	300	500
SECONDARY STRUCTURE PROBLEMS?	YES	LESS	LESS

Conditions to optimize double-stranded DNA sequencing were examined. A wide variety of conditions were investigated including quality of template, denaturation of duplex DNA, annealing conditions, sequencing reaction conditions, gel electrophoresis conditions and sequencing enzyme used. Three enzymes were tested which included the Klenow fragment of *E. coli* DNA polymerase I, avian myeloblastosis virus (AMV) reverse transcriptase, and T7 DNA polymerase (Sequenase). Sequenase was superior to Klenow and AMV reverse transcriptase since using this enzyme reduced reaction time, amount of radiolabeled nucleotide, and enabled sequencing through DNA secondary structure and more nucleotides to be read from a single reaction.

cDNA in 10 mM Tris pH 8.0, 50 mM KCl, 2.5 mM MgCl₂, each primer at 1 μ M, each dNTP (dATP, dCTP, dGTP, TTP) at 200 μ M and 2.5 units of polymerase. The samples were overlaid with -100 μ l of mineral oil to prevent evaporation and subjected to 25-30 cycles of amplification as described. After the last cycle, all samples were incubated for an additional 7 minutes at 72°C to ensure that the final extension step was complete. After ethanol precipitation and resuspension in 100 μ l TE buffer, samples were resolved on 4% Nusieve or 2.5% agarose gels. Southern transfers were performed using nylon membranes.

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